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Phosphatase Epsilon in Mammary Tumor Cell Lines

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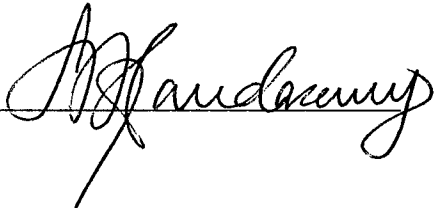
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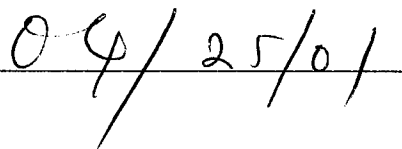
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13. ABSTRACT (Maximum 200 Words) Studies included in this report describe establishing Neu-induced mammary tumor cell lines, which either do or do not express the protein tyrosine phosphatase epsilon (PTPe), for the purpose of identifying substrates of the phosphatase. These cells display altered morphology and altered growth and adhesion properties, supporting our previous results that PTPe plays an accessory role in Neu- and Ras-induced transformation of mammary epithelial cells. We provide evidence to show that phosphorylation of the key kinase Src is altered in tumor cells lacking PTPe; changes of this type have been shown to inactivate Src in other systems. We provide further evidence to show that altered Src phosphorylation is the result of lack of PTPe activity and not of other undetermined properties of PTPe-deficient tumor cells. In a study related to our previous demonstration that the delayed-rectifier, voltage-gated potassium channel Kv2.1 is a physiological substrate of PTPe, we show here that tyrosine residue 124 is the site of phosphorylation of this channel by Src and of dephosphorylation by PTPe. As such, this study strongly supports this channel being regulated by the mutually-antagonistic activities of Src and PTPe.				
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4. Introduction:

Our research focuses on providing molecular-level understanding the role of protein tyrosine phosphatase epsilon (PTPe) in the genesis of breast cancer by identifying physiological substrates of the enzyme. We have previously demonstrated a link between PTPe and genesis of mammary tumors in mice *in vivo*; the project supported by the USAMRMC is aimed at taking these findings one step further by defining what is one of the most basic items of information needed to understand the roles of PTPe - the identities of its substrates. We aim to achieve this goal through use of substrate-trapping methodology, i.e., by use of PTPe mutants which have lost most of their catalytic ability but are able to bind their phosphorylated substrates and remain bound to them during purification. Specifically, in the course of this project we will construct and characterize the necessary mutant PTPe molecules, identify proteins which bind PTPe substrate-traps or verify that candidate substrates of PTPe indeed bind substrate-trapping mutants of PTPe, verify that molecules which bind are indeed substrates of PTPe, and examine how dephosphorylation by PTPe affects their function. The first annual progress report, submitted last year (December 1999), described the construction of substrate-trapping mutants of PTPe and preliminary work we had done with them. That study resulted in identification of voltage-gated potassium channels as physiological substrates of PTPe, and documented how aberrant phosphorylation of such channels in mice lacking PTPe results in myelination abnormalities in the peripheral nervous system. Studies performed this year have focused on the Src tyrosine kinase. We present data which strongly indicates that this kinase is a substrate of PTPe in mammary tumors initiated by the Neu oncogene.

5. Body of report:

Studies planned for the period covered by this report:

The original grant proposal outlined for months 13-24 of the funding period included isolation of PTPs substrates, verification that the isolated molecules are indeed substrates of PTPe, resource building (such as generation of antibodies and other reagents, if not commercially available), and beginning of analysis of the physiological meaning of these molecules being substrates of PTPe. A major conclusion from last year's research, which was included in last year's report, was to place more emphasis on the "educated guess" approach to finding substrates of PTPe, and to use substrate-trapping mutants of PTPe in this context to verify that candidate substrates bind these mutants. It is believed

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that this approach would save significant time and would streamline the process of actually getting to the physiologically-relevant part of the study, which is its core. As we suggested last year, we focused our efforts this year on Src, a tyrosine kinase which regulates a variety of cellular activities. This kinase has been shown to be a substrate of PTPalpha (PTPa), a phosphatase related to PTPe but whose relevance to breast cancer is not well established (Zheng et al., 1992; den Hertog et al., 1993; Su et al. 1999; Ponniah et al., 1999; Ardini et al., 2000). The known functions of Src in transforming cells and in regulating other major cellular processes suggested that the physiological ramifications of Src being a substrate of PTPe would be significant.

Studies performed during the period covered by the report:

Src, PTPe, and mammary tumors:

A. Establishing PTPe-deficient mammary tumor cell lines

Key reagents involved in this year's study were PTPe-knockout mice, which had been prepared as part of a study outside the scope of this report (Peretz et al., 2000). In a study unrelated to the present project, these mice had been mated with MMTV-Neu transgenic mice, in which the Neu transgene was expressed in the mammary epithelium due to the activity of the MMTV (Mouse Mammary Tumor Virus) promoter/enhancer, giving rise to mammary tumors (Muller et al., 1988). We had previously established that the receptor-type form of PTPe (tm-PTPe) plays an accessory role in this process, as mammary tumors induced specifically by either Neu or Ras exhibit significant upregulation of tm-PTPe (Elson and Leder, 1995a). Furthermore, transgenic mice expressing tm-PTPe in their mammary epithelium develop mammary hyperplasia and associated tumors (Elson, 1999). The mating described above was performed in order to examine whether the role tm-PTPe plays in this process is necessary for the ability of Neu to transform mammary epithelial cells.

Neu-induced tumor incidence in mice was similar irrespective of whether the mice did or did not contain a functional PTPe gene. We present the tumor-free survival curve of these mice as Figure 1 for purpose of clarity, even though this result is not part of the study being reported here. However, we reasoned that substrates of PTPe could be phosphorylated differently in Neu-induced mammary tumors, dependent upon whether PTPe was or was not expressed in these tumors. We further reasoned that cell lines derived from these tumors would be valuable assets in identifying substrates of PTPe by

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either the "educated guess" or the substrate-trapping approach.

In order to examine this possibility, we established several of the aforementioned mammary tumors in culture as cell lines. Lines were established by isolating tumor tissue from mice, finely mincing it in tissue culture dishes in culture medium (DMEM/10% bovine serum/ 2mM Glutamine/ 100 units/ml penicillin/ 100 µg/ml streptomycin), and passaging the cells until a healthy population of uniform epithelial morphology arose. The cell lines in question all arose on the same genetic background (50% FVB/N, 50%C57Bl6/129) and all were transformed due to the activity of the Neu protein, as no tumors arose in mice which did not carry the MMTV-Neu transgene (not shown). However, with respect to PTPe, three groups of tumors could be distinguished - tumors which contained both alleles of PTPe (wild-type, WT), tumors which arose in mice heterozygous for the PTPe null alleles, and tumors which arose in mice lacking both alleles of PTPe mice. Protein blots documenting the Neu and PTPe status of several tumor cell lines used in this study are shown in Figure 2.

B. Properties of PTPe-deficient mammary tumor cells

Although the cellular morphology of tumor tissue obtained from mice of all three PTPe genotypes did not vary significantly when analyzed as tumor tissue (not shown), the appearance of cell lines derived from similar tumors was clearly dependent upon presence or lack of PTPe. As seen in Figure 3, cells from tumors which arose in WT mice (i.e., mice which expressed the Neu transgene and the normal WT complement of PTPe) were epithelial in morphology, possessed prominent nuclei, and exhibited compact, cobblestone-like morphology. In contrast, cells from similar tumors which arose in mice lacking both alleles of PTPe were much more flat and spread out, although they were also of clear epithelial morphology. Anecdotal evidence, which is currently in the process of being verified and quantified, indicates that PTPe-deficient tumors proliferate more slowly and adhere to tissue-culture plates less strongly than WT or PTPe heterozygous tumor cells.

C. Src phosphorylation in PTPe-deficient mammary tumor cells

The belief that Src might be a physiological substrate of PTPe in mammary tumor cells led us to ask whether Src phosphorylation and activity is altered in tumor cells lacking PTPe. Phosphorylation of two key tyrosines in Src is associated with Src activity- Y527 and Y418. Once phosphorylated, pY527 binds the SH2 domain of Src,

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thereby "locking" the molecule in an inactive form. Upon dephosphorylation of pY527, Src molecules change their conformation and become active, autophosphorylating Y418 in the process. Phosphorylation of Y527 and hypophosphorylation of Y418 have been associated repeatedly with reduced Src activity (Abram and Courtneidge, 2000).

We examined the phosphorylation state of Src at these two tyrosine residues in mammary tumor cell lines using commercially-available anti-phospho-specific antibodies directed at Y527 (anti-pY527Src) or Y418 (anti-pY418Src). Experiments consisted of subjecting tumor cell lysates to SDS-PAGE and protein blotting with either of the two phospho-specific antibodies, after which the signal obtained was normalized to the total amount of Src present in the cell lysates. The latter information was obtained by probing the protein blots with an anti-Src antibody, which is not phospho-specific. As seen in Figure 4, clear-cut differences in phosphorylation of Src were observed as a function of the PTPe genotype of the tumor cells. PTPe-deficient tumor cells exhibited higher phosphorylation at Y527 and reduced phosphorylation at Y418 as compared with WT tumor cells. This pattern of phosphorylation is consistent with Src activity being lower in PTPe-deficient tumor cells; Src activity in both cell types is currently being examined.

D. Src phosphorylation in cells expressing exogenous PTPe.

In order to verify that alterations in Src phosphorylation were a direct result of lack of PTPe activity in the mammary tumor cell system, we examined Src phosphorylation in cells which we had transfected with PTPe. Preliminary experiments were performed in NIH3T3 fibroblasts, and consisted of examining Src phosphorylation following transfection with various forms of PTPe. Experiments of the type described above using anti phospho-Src-specific antibodies revealed that expressing PTPe reduced phosphorylation of Src at Y527, although the magnitude of this effect was weak; no effect was noted at Y418 (not shown). However, transfection efficiency in these cells was relatively low by both the calcium phosphate and by the Lipofectamine procedures. As our phospho-specific antibody approach relies on examining phosphorylation of Src from all cells present on a plate, we feared that the effect we might be generating in Src present in transfected cells might be diluted by Src phosphorylation not changing in the majority of the cells, which were non-transfected.

In order to bypass this difficulty we turned to cells which lack Src, Fyn, and Yes

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(SYF cells, Klinghoffer et al., 1999). We reasoned that upon transfection with Src and PTPe, Src would be present only in transfected cells together with PTPe, thereby maximizing our chances of detecting any effects PTPe might have on Src. Indeed, this series of experiments revealed that expressing PTPe reduced phosphorylation of Src at Y527 and somewhat increased it at Y418, arguing that PTPe does indeed dephosphorylate and activate Src (Figure 5). Side-by-side comparison of the two forms of PTPe with each other and with PTPa revealed that the cytoplasmic form of PTPe (cyt-PTPe) was most efficient at targeting Src, although both forms of PTPe seemed more active in this respect than PTPa (Figure 5).

Of particular interest is the finding that, while PTPa reduces phosphorylation at Y527 and increases phosphorylation at Y418, cyt-PTPe and tm-PTPe seems to reduce phosphorylation at Y527 while not affecting Y418 (Figure 5). We tentatively explain this result at present as arising from the fact that PTPa acts relatively weakly on Src and therefore limits its activity to Y527. Upon dephosphorylation of Y527 in Src, the kinase becomes active and autophosphorylates at Y418. In contrast, increased activity of cyt-PTPe and tm-PTPe towards Src results in dephosphorylation of Y527, and in some PTPe activity "spilling over" and dephosphorylating Y418 as well. Data consistent with this interpretation was obtained upon examining the effects of a membrane-targeted version of cyt-PTPe. As expected, this molecule, in which a myristylation signal was added at its extreme N-terminus, was extremely active towards Src, and reduced phosphorylation at both Y527 and Y418 to the limits of detection of our anti-phospho-Src antibodies (Figure 5).

E. Substrate trapping studies in PTPe-deficient mammary tumor cell lines.

We have attempted to transfect mouse mammary tumor cell lines with expression vectors of our making using the calcium phosphate or Lipofectamine techniques. Substrate-trapping studies require high transfection yields; in contrast, these transfection experiments have shown that this class of cells is typically transfected at low efficiency. In order to circumvent this problem we are now in the final stages of constructing vectors for retroviral-mediated infection of these tumor cells. The major advantage of infection is that it assures extremely high success rates in introducing exogenous DNA into cells, hence we hope it will allow us to perform substrate-trapping studies in these mammary tumor cells, which are otherwise ideal for this purpose.

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Discussion:

The study described above provides strong evidence that tm-PTPe is a physiological regulator of Src in mammary tumor cells. As indicated above, our previous studies established tm-PTPe as an accessory factor in Neu- and in Ras-mediated transformation of mouse mammary epithelial cells. This conclusion is based on up-regulation of tm-PTPe expression in such tumors (Elson and Leder, 1995a), and on the mammary hyperplasia and tumorigenesis phenotype of transgenic mice, which overexpress tm-PTPe in their mammary epithelium (Elson, 1999). The present study shows that cells derived from mammary tumors initiated by Neu, but which lack tm-PTPe, are "less well off" in the sense that they are more slow to divide, appear to be less adherent in culture, and exhibit altered morphology. These results clearly show that, irrespective of the molecular mechanism involved, tm-PTPe does in fact perform an important role in maintaining the transformed phenotype of Neu-induced mammary tumor cells.

Studies presented here also suggest a clear molecular-level explanation for the altered properties in PTPe-deficient cells. Altered growth rates, morphology, and adherence properties have all been previously linked with abnormal Src activity. Indeed, our Src phosphorylation results are very suggestive that Src activity is reduced in PTPe-deficient mammary tumor cells; this issue will be addressed experimentally in the coming year, as outlined below.

In all, the data suggest that inactivating tm-PTPe might have beneficial effects in terms of halting growth of Neu-induced mammary tumor cell lines. Were Src indeed shown to be the vehicle by which PTPe exerts its effects, one could presumably achieve the same results by other means, which are currently available, such as pharmacological inhibition of Src (Blake et al., 2000). The central role of Neu in human mammary tumorigenesis and of Src in regulating a multitude of cellular processes underscore the importance of this finding.

Our plans for the coming year include:

- * Measuring Src kinase activity in the above set of Neu-induced mammary tumor cell lines, to determine whether the changes in Src phosphorylation are in fact correlated with the expected reduction in Src activity in cells lacking PTPe.
- * Overexpressing the substrate-trapping mutants of PTPe in these tumor cells by retroviral-mediated infection, and searching for molecules which bind these mutants.

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* Determining whether substrate-trapping mutants of Src can bind and precipitate Src in transfected cells and in Neu-induced mammary tumors.

* Determining whether PTPe-deficient mammary tumor cell lines are indeed defective in adhesion, as preliminary data suggest. We will quantify cell detachment and migration rates, as well as the phosphorylation and activity of focal adhesion kinase (FAK), a kinase important for adhesion and which is known to be regulated by Src.

* We will determine if the combined phenotype of PTPe-deficient mammary tumor cells (slow growth, decreased adhesion, altered morphology) is due to altered Src phosphorylation (and presumably reduced Src activity), or are unrelated to changes in Src. This will be done by transfecting PTPe-deficient mammary tumor cell lines with a constitutively-active mutant of Src (Y527F Src), thereby adding Src activity to these cells in a manner which is likely not affected by lack of PTPe. We will then examine the above phenotypic parameters; were they to change such that they resemble more these properties in PTPe-expressing, Neu-induced mammary tumors, this would indicate that the phenotype observed in PTPe-deficient tumor cells are indeed due to changes in Src. This experiment assumes that the above phenotypic changes are reversible.

PTPe and the Kv2.1 potassium channel

F. Identification of the site of dephosphorylation by PTPe in Kv2.1

As indicated above, the first practical use to which the substrate-trapping mutants of PTPe were put to use in our hands was to identify the delayed-rectifier, voltage-gated potassium channel Kv2.1 as a physiological substrate of PTPe (Peretz et al., 2000). During the past year we identified tyrosine residue 124 of rat Kv2.1 as the tyrosine residue which is targeted by Src and PTPe.

Our previous data indicated that in dephosphorylating Kv2.1, cyt-PTPe was in effect countering the activity of Src towards this channel molecule. Kv2.1 has 19 tyrosine residues, of which 14 are in parts of the molecule which are cytoplasmic, i.e., to which Src would be expected to have access. Furthermore, the consensus sequence for phosphorylation by Src is known to be XEIIYXE, corresponding nicely with the sequence present at positions 121-126 of rat Kv2.1 (DEIYLE, Sobko et al., 1998). The data suggested that the tyrosine targeted by PTPe would probably be part of a Src consensus sequence, hence we decided to examine the possibility that Y124, the only tyrosine present in the above sequence, was the substrate of both Src and PTPe. In order

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to achieve this goal, we mutated Y124 in Kv2.1 to an unphosphorylatable phenylalanine residue.

As seen in Figure 6, Y124F Kv2.1 was significantly less phosphorylated by Src in intact cells. Phosphorylation was reduced by approximately 60%, indicating that this residue is indeed the major site of phosphorylation by Src in Kv2.1. Co-expressing wild-type PTPe together with Src in cells expressing either wild-type or Y124F Kv2.1 reduced Kv2.1 phosphorylation to similar background levels, indicating that the residual 40% of Src-mediated phosphorylation in Kv2.1 was also a target of PTPe (not shown)

Further studies indicated that the Y124F mutant of Kv2.1 was significantly less able than wild-type Kv2.1 to bind the D-to-A substrate-trapping mutant of cyt-PTPe (Figure 7). Binding was reduced by ~65%, i.e. by approximately the same extent that phosphorylation by Src of Y124F Kv2.1 was reduced. This last result is crucial, as it directly shows that Y124 is the major site to which the active site of PTPe binds, consistent with PTPe-mediated dephosphorylation occurring at this site.

Discussion:

Our studies have tentatively identified Y124 of Kv2.1 as the residue which is phosphorylated by Src and which is dephosphorylated by PTPe. This result is still being viewed by us as tentative pending electrophysiological activity measurements of wild-type and Y124F Kv2.1, which will be performed in the near future and which are outlined below. We expect Y124F Kv2.1 to be significantly less active following cellular depolarization than wild-type Kv2.1. Were Y124 the site of action by both enzymes on Kv2.1, this would provide a much-needed "molecular address" for the site of their mutually-antagonistic activities towards the channel protein. Furthermore, Src and PTPe have each been identified as acting predominantly on Y124 of Kv2.1 in an independent series of experiments, hence this result is further support for the notion that Kv2.1 is a common substrate for both enzymes (Peretz et al., 2000).

In addition to verifying that the Y124F mutation affects Kv2.1 activity, we will also transfect this mutant of Kv2.1 into wild-type Schwann cells and estimate its degree of tyrosine phosphorylation by SDS-PAGE and protein blotting. This experiment is important in allowing us to determine whether the ~35% of Src-mediated tyrosine phosphorylation and substrate-trap binding we do see in Y124F Kv2.1 is real or is an artifact of exogenous expression of Src in the cells in which the experiment was done. If

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this is so, we expect residual tyrosine phosphorylation of Y124F Kv2.1 in Schwann cells to be negligible.

Our immediate plans for the coming year in this project include therefore:

- * Demonstrating whether Y124F Kv2.1 is less or totally non-functional in comparison with wild-type Kv2.1. This will be determined by whole-cell patch clamp experiments in transfected cells, much as we have done in the past (Peretz et al., 2000).
- * Determining whether the residual 35% of phosphorylation present on Kv2.1 is real or is an artifact of high Src expression levels in transfected cells. For this purpose we will isolate Schwann cells from mice and express either wild-type or Y124F Kv2.1 separately in them. Each Kv channel will be immune-precipitated from the cells via a FLAG tag added to its amino terminus, and its tyrosine phosphorylation level will be estimated by SDS-PAGE/protein blotting analyses.

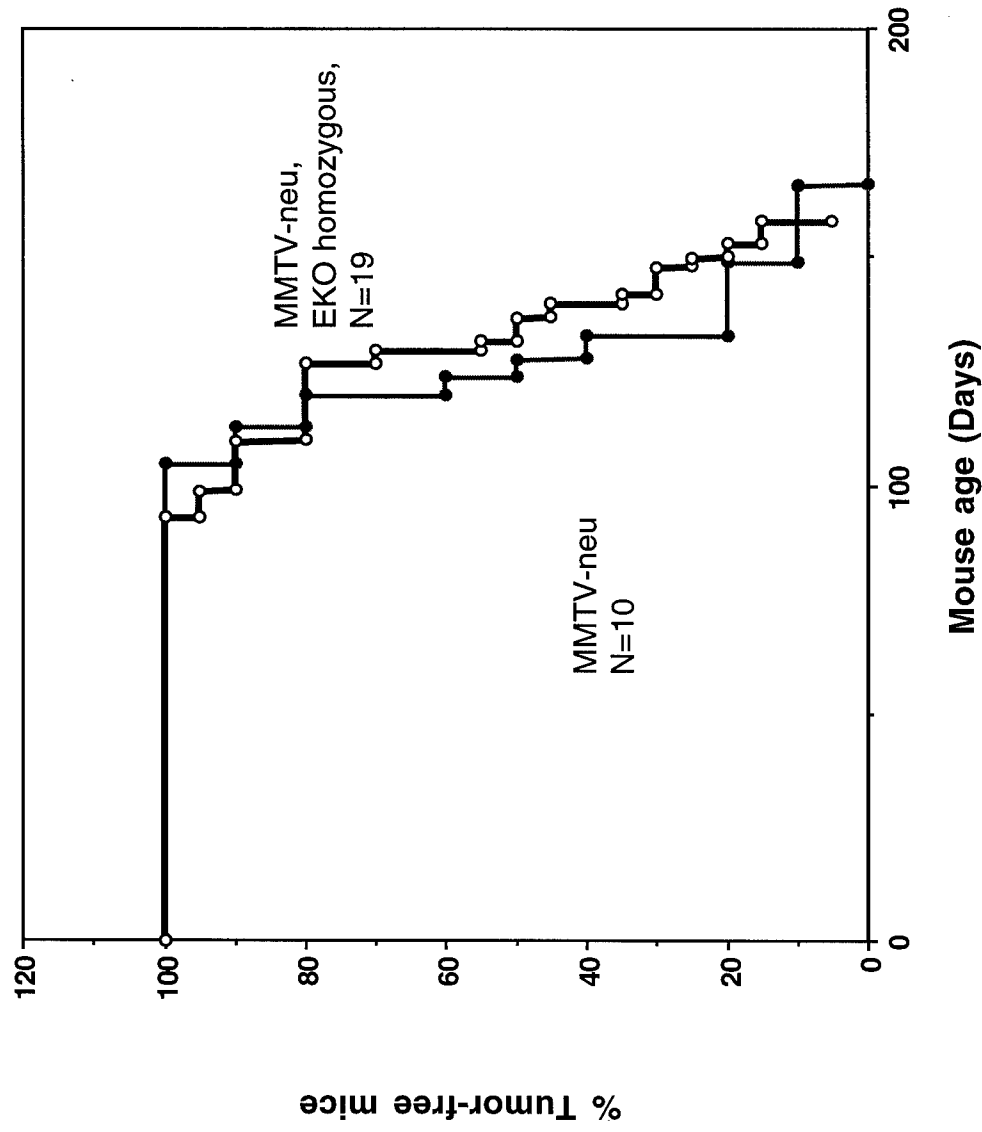


Figure 1: PTPe deficiency does not alter kinetics of tumor formation by the MMTV-activated neu transgene. Female mice of the indicated genotypes were allowed to mate at will and were followed for tumor appearance. Shown is the percentage of tumor-free surviving mice as a function of their age.

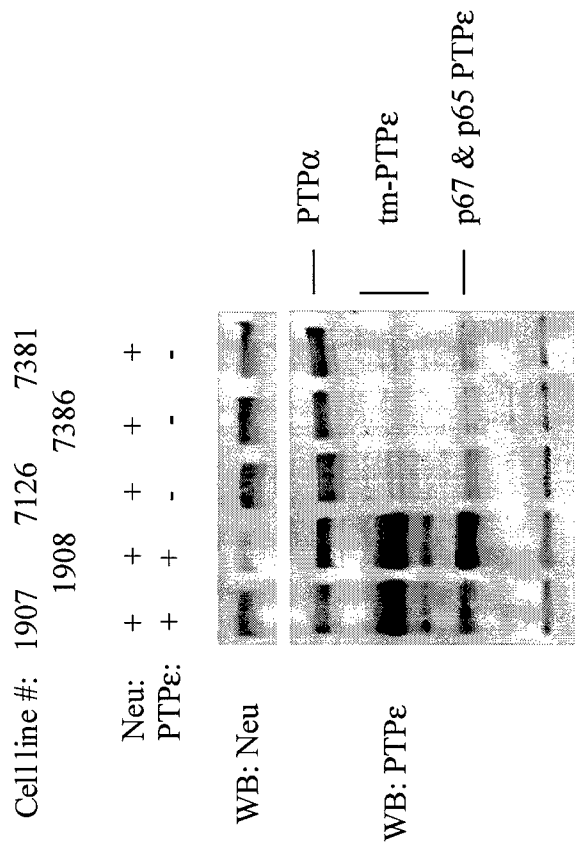


Figure 2: Expression levels of Neu and PTPe in tumor cell lines derived from Neu-based mammary tumors of wild-type or of PTPe-deficient mice. Total protein extracts were prepared from cell lines of the indicated genotypes. Following SDS-PAGE analysis, blots were probed with anti Neu (top panel) or anti-PTPe antibodies (bottom panel). Note that the anti-PTPe antibody cross-reacts with PTPα, levels of which are unchanged in PTPe-deficient tumors. p67 and p65 PTPe are shorter forms of PTPe which are produced by internal initiation of translation of PTPe mRNA (p67) or from proteolytic cleavage of full-length PTPe (p65; Gil-Henn et al., 2000).

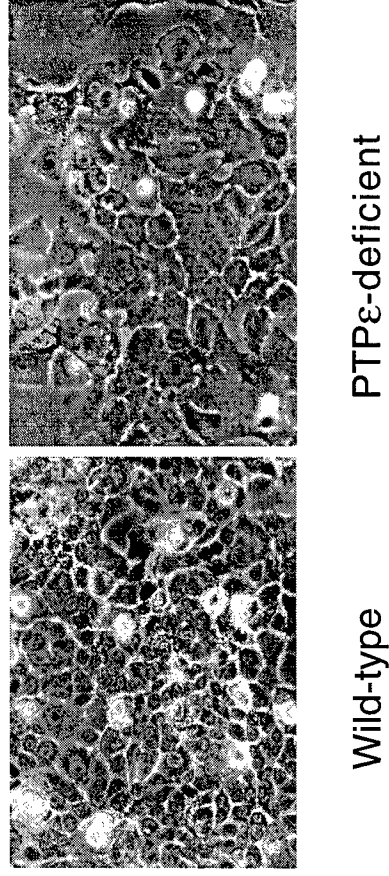


Figure 3: Morphology of typical wild-type and PTPe-deficient, Neu-induced mouse mammary tumor cell lines. Cells were visualized by phase-contrast microscopy. Lines photographed are 1908 (wild-type) and 7386 (PTPe-deficient). Note less compact and more spread-out appearance of PTPe-deficient cells. Results are typical of all cells of a given genotype. Cell lines heterozygous for PTPe appeared had wild-type morphology.

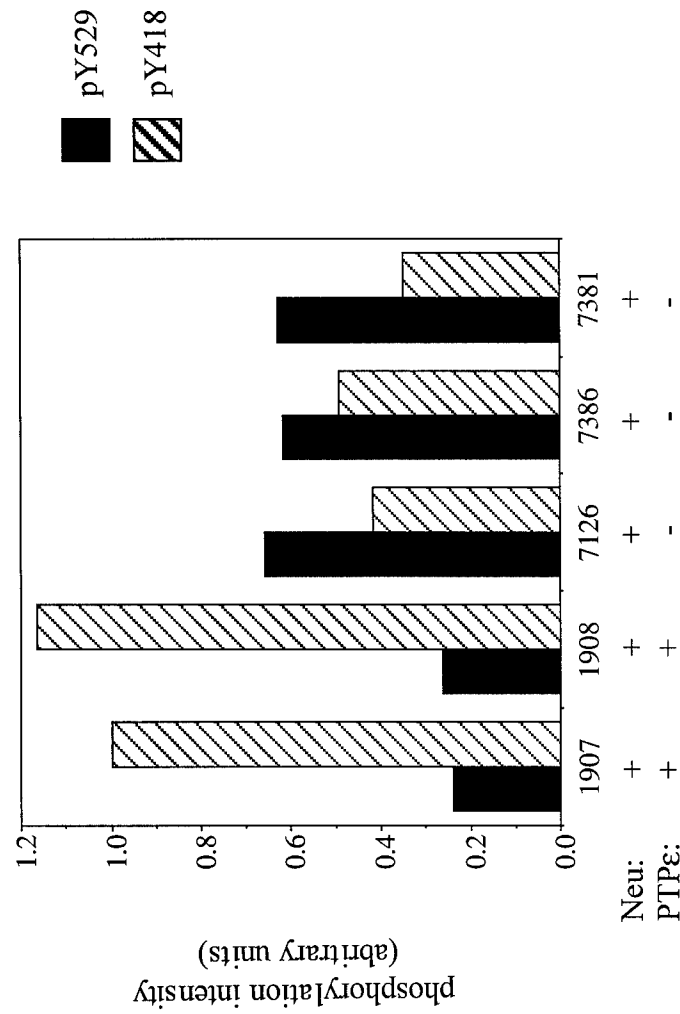


Figure 4: Presence of absence of PTPe affects Src phosphorylation in Neu-based mammary tumor cells. Lysates of the indicated cell lines were analyzed by SDS-PAGE and protein blotting, and were probed with antibodies specific for either phospho-Y527 Src or phospho-Y418 Src. Resulting signals were quantified using a scanning densitometer and normalized to Src expression levels in the cells, determined by probing identical blots with pTyr-insensitive anti-Src antibodies. All cells expressed similar levels of total Src. Graph depicts results of a single experiment, representative of three performed.

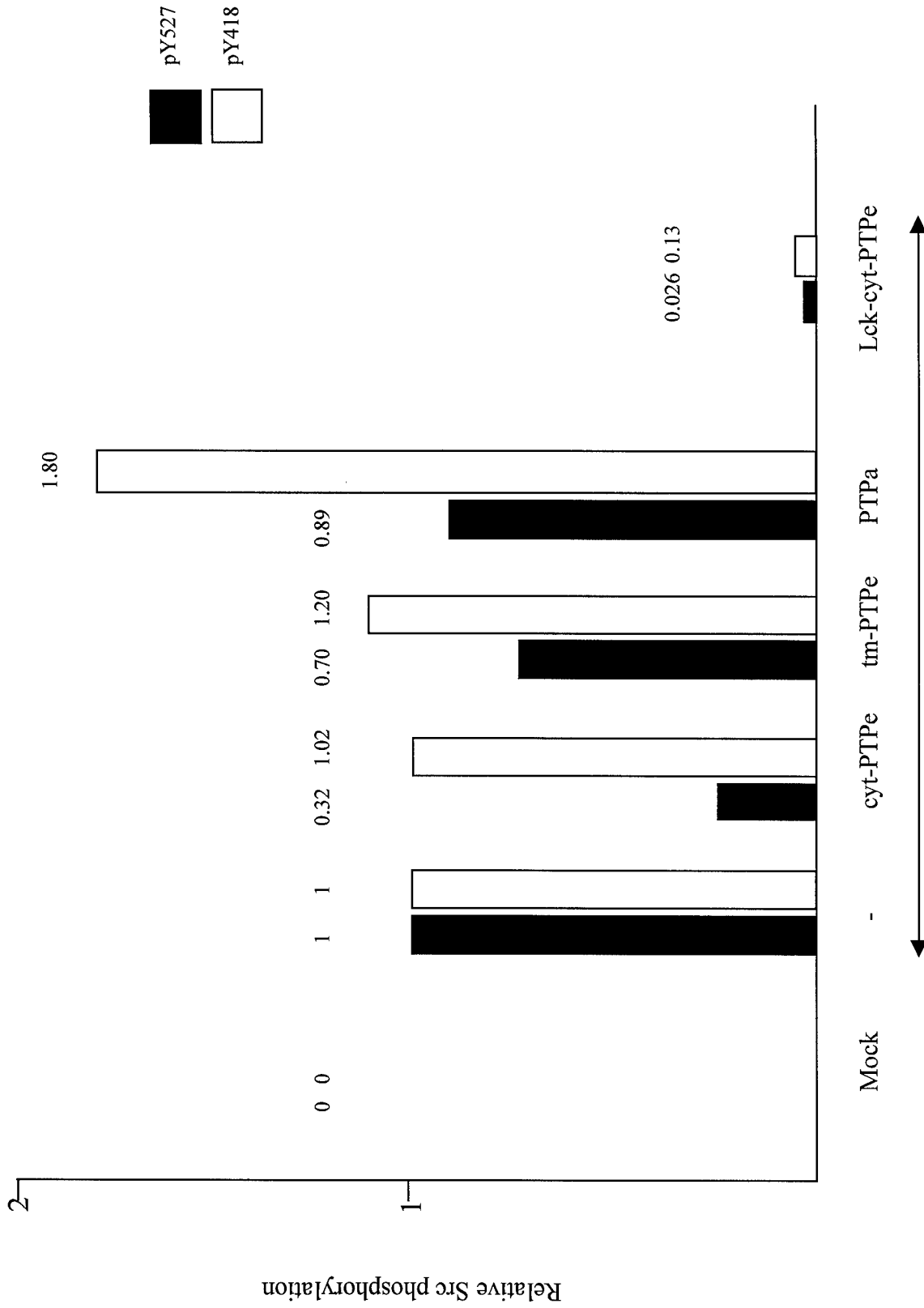


Figure 5: Phosphorylation of Y527 and Y418 of Src in SYF cells following co-expression with various forms of PTPe. Abbreviations for the various forms of PTPe are: cyt-PTPe - non-receptor form; tm-PTPe - non-receptor form; Lck-cyt-PTPe - non-receptor form of PTPe with an Lck myristoylation signal at its amino terminus. PTPa- protein tyrosine phosphatase alpha. Fold changes in tyrosine phosphorylation, relative to Src phosphorylation in the absence of PTPe, are shown above each bar.

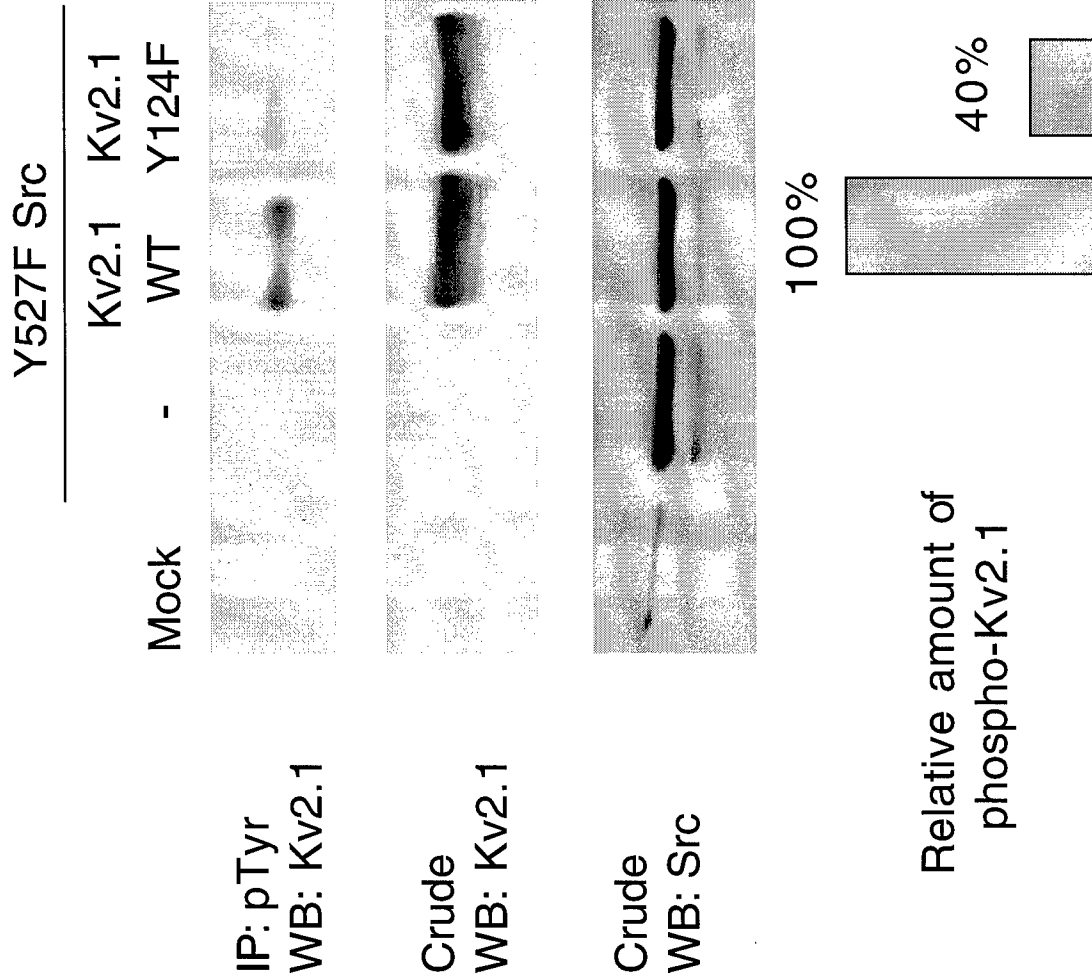


Figure 6: Reduced Src-mediated phosphorylation of Y124F Kv2.1 in comparison with wild-type Kv2.1. 293 cells were transfected with activated (Y527F) Src and wild-type or Y124F Kv2.1 as indicated. Phosphorylated proteins were immunoprecipitated from cell lysates with anti-phosphotyrosine antibodies, and the amount of Kv2.1 in the immunoprecipitates was estimated using anti-Kv2.1 antibodies. Phosphorylation of Y124F Kv2.1, normalized to Kv2.1 expression levels in the cells, was reduced by 60% as compared with wild-type Kv2.1. Shown are results of a single experiment, representative of two performed.

Kv2.1: - WT Y124F - WT Y124F

D245A cyt-PTPε: - - - + + + +

IP: PTPε/WB: Kv2.1

IP: PTPε/WB: PTPε

Crude/WB: Kv2.1



Figure 7: Y124F Kv2.1 binds less of the D245A (substrate-trapping) mutant of cyt-PTPε. 293 cells were transfected with activated D245A cyt-PTPε and wild-type or Y124F Kv2.1 as indicated. PTPε was immunoprecipitated from cell lysates with anti-FLAG antibodies, and the amount of Kv2.1 in the immunoprecipitates was estimated using anti-Kv2.1 antibodies. Binding of Y124F Kv2.1, normalized to Kv2.1 expression levels in the cells, was reduced by 65% as compared with wild-type Kv2.1.

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6. Key Research Accomplishments:

- * Establishing a series of PTPe-deficient and PTPe-expressing, Neu-induced mouse mammary tumor cell lines.
- * Use of said cell lines to demonstrate that lack of PTPe affects cell morphology and growth rates in a way which argues that PTPe is required for maintaining transformed phenotype.
- * Use of said cell lines to show that lack of PTPe affects Src phosphorylation in a manner consistent with reduced activity of Src.
- * Demonstration that overexpression of PTPe affects Src phosphorylation in a manner opposite to that seen in PTPe-deficient tumor cell lines. Tentative identification of Src as a physiological substrate of PTPe.
- * Identification of Y124 of the Kv2.1 potassium channel as the site phosphorylated by Src and dephosphorylated by PTPe.

7. Reportable Outcomes:

Manuscripts:

1. Peretz, A*., Gil-Henn, H*., Sobko, A., Shinder, V., Attali, B and Elson, A. (2000) - Hypomyelination and increased activity of voltage-gated potassium channels in mice lacking protein tyrosine phosphatase ϵ . *EMBO J. 19 (15) 4036-4045* (* - equally contributing first authors).
(listed as in preparation in previous report).

2. Tiran, Z., Peretz, A., Gil-Henn, H., Attali, B. and Elson, A. - Tyrosine 124 of the delayed-rectifier, voltage-gated potassium channel Kv2.1 is phosphorylated by Src and dephosphorylated by PTP epsilon in vivo (*in preparation*).

Abstracts/presentations:

1. Second International Conference on Signal Transduction (ICST), May 2000, Dubrovnik, Croatia.
PTP Epsilon is required for myelination of peripheral nerves in early post-natal mice.

Ari Elson, Bernard Attali, Asher Peretz, Hava Gil-Henn, Alex Sobko, and Vera Shinder.

(Oral presentation)

2. FASEB meeting on protein phosphorylation. July 2000, Copper Mountain, Colorado, USA.

PTP Epsilon is required for myelination of peripheral nerves in early post-natal mice.

Ari Elson, Bernard Attali, Asher Peretz, Hava Gil-Henn, Alex Sobko, and Vera Shinder.

(Oral presentation)

3. Era of Hope meeting, May 2000, Atlanta, USA.

Tyrosine dephosphorylation and cellular regulation: PTP epsilon down-regulates voltage-gated potassium channels in vivo.

Ari Elson, Asher Peterz, Hava Gil-Henn, Alex Sobko, and Bernard Attali.

(Poster presentation).

4. In addition, lectures on the Kv2.1 project were delivered by Dr. Elson during the past year at the following venues:

- * Department of Genetics, Hebrew University of Jerusalem, Israel

(December, 1999)

- * Department. of Orthopaedics, Yale Medical School (May2000)

- * Department of Genetics, Harvard Medical School (July 2000)

- * Department of Cell Research and Immunology, Tel Aviv University

(November, 2000)

Patents and Licenses:

N/A

Degrees obtained due to support by this grant:

None (yet: grant is supporting work of individuals listed below, although they have not yet obtained their degrees).

Development of cell lines:

In the course of work described here we have produced several mouse mammary tumor cell lines. All of these lines are transformed due to the activity of the Neu oncogene. Three cell lines are PTPe-deficient, two are heterozygous for the PTPe null allele, and two are wild-type with respect to PTPe.

Informatics:

N/A

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Funding applied for based on work supported by this award:

Based on the potassium channel work performed last year we have sought and obtained funding from the Israeli Academy of Sciences to pursue this topic further. Brunt of further research on this topic, which is now breaking away from substrate-trapping technology and from breast cancer in general, will be funded by this new grant (funding has been granted at \$50,000/year for four years).

Based on our results concerning regulation of Kv channels by PTPe, we have approached the Human Frontiers Research Program and the United States-Israel Binational Science Foundation for funding to study regulation of these channels by the related PTP alpha, using, among other reagents, PTP alpha-deficient mice we have obtained from collaborators.

Employment and research opportunities supported by this grant:

Funds support work performed by two PhD students in the lab - Ms. Hila Toledano-Katchalski (who also receives a stipend funded from this grant), and Ms. Hava Gil-Henn.

8. Conclusions:

A. Src as a possible substrate of PTPe in mammary tumor cells:

This study provides strong evidence that tm-PTPe is a physiological regulator of Src in mammary tumor cells. Mammary tumor cells, which lack the receptor-type form of PTPe, exhibit changes in Src phosphorylation, which have been shown in other systems to correlate with reduced activity of the kinase. Furthermore, the morphology, growth rates, and adhesion properties of PTPe-deficient mammary tumor cells are in line with what might be expected of cells with reduced Src activity. This result is in excellent agreement with our previous studies, which have been outlined in the main part of this report, and which have established that the receptor-type PTPe is an accessory factor in the processes by which Neu or Ras transform mammary epithelial cells (Elson and Leder, 1995; Elson, 1999).

Additional results which we have obtained in SYF cells strongly suggest that the changes in Src phosphorylation are directly related to PTPe activity. The changes observed in this respect in the PTPe-deficient tumor cells are therefore likely to result from lack of PTPe activity, rather than, for example, Neu targeting a different subtype of

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mammary epithelial cells in PTPe-deficient mice as compared to wild-type mice. Our studies have also demonstrated that both major forms of PTPe, receptor-type and the non receptor-type PTPe, act upon Src in a manner somewhat stronger than the related PTPa, and that membrane association plays an important factor in allowing PTPe to act upon Src. The latter point was demonstrated by the significantly increased activity of the non-membranal form of PTPe towards Src, following tethering it to the membrane via addition of a myristylation signal.

Practical benefits from these findings include firm *in vivo* support for the accessory role of receptor-type PTPe in mammary transformation by Neu. This finding is a solid one, irrespective of whether the molecular mechanism by which it is brought about will turn out to be via regulation of the Src kinase. This finding is important, as PTPe appears to be associated specifically with mammary transformation by Neu or Ras, the former of which is known to be a major factor in human breast cancer. Furthermore, these studies suggest possible points for therapeutic intervention by inhibiting PTPe activity. If the effects of PTPe on mammary tumor cells turn out to be mediated via the activity of PTPe on Src, as we believe they are, an alternative therapeutic option would be to inhibit Src. This latter strategy is more straightforward, as Src is an intensively studied enzyme for which inhibitors are available (e.g., Blake et al., 2000).

B. Y124 of Kv2.1 as the site of action of Src and PTPe:

This study has resulted in the tentative identification of Y124 of Kv2.1 as the residue which is phosphorylated by Src and which is dephosphorylated by PTPe. Although, as explained in the main body of this report, we still view this result as being somewhat tentative pending completion of ongoing electrophysiological experiments, the biochemical part of the study is complete. Based on biochemical data, we have been able to show that indeed Y124 of Kv2.1 is a substrate simultaneously of Src, which phosphorylates it, and PTPe, which dephosphorylates it and thereby counters the activity of Src. This is further proof of the model we proposed (Peretz et al., 2000), by which Src and PTPe are a kinase/phosphatase pair which antagonize each others function in regulating Kv channel activity.

This study has pinpointed the precise site where most of the regulation of Kv2.1 by Src and PTPe takes place. Since Kv channels are a large family of structurally-similar molecules, this finding could be applicable to other channel molecules as well. Expression of Kv channels is widespread and is involved in the function of many organ systems. We

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have previously demonstrated that lack of PTPe activity affects myelination of peripheral nerve axons, most likely by altering the phosphorylation and activity of Kv channels (Peretz et al, 2000). Our current results improve our understanding of how this important class of molecules is regulated and might in the future open therapeutic windows for treating severe diseases which affect myelination, such as multiple sclerosis.

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10. Appendices:

Three copies of the following paper are included with this report:

Peretz, A., Gil-Henn, H., Sobko, A., Shinder, V., Attali, B and Elson, A. (2000) -
Hypomyelination and increased activity of voltage-gated potassium channels
in mice lacking protein tyrosine phosphatase ϵ . *EMBO J.* 19 (15) 4036-4045

Hypomyelination and increased activity of voltage-gated K⁺ channels in mice lacking protein tyrosine phosphatase ϵ

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A.Peretz and H.Gil-Henn contributed equally to this work

Protein tyrosine phosphatase epsilon (PTP ϵ) is strongly expressed in the nervous system; however, little is known about its physiological role. We report that mice lacking PTP ϵ exhibit hypomyelination of sciatic nerve axons at an early post-natal age. This occurs together with increased activity of delayed-rectifier, voltage-gated potassium (Kv) channels and with hyperphosphorylation of Kv1.5 and Kv2.1 Kv channel α -subunits in sciatic nerve tissue and in primary Schwann cells. PTP ϵ markedly reduces Kv1.5 or Kv2.1 current amplitudes in *Xenopus* oocytes. Kv2.1 associates with a substrate-trapping mutant of PTP ϵ , and PTP ϵ profoundly reduces Src- or Fyn-stimulated Kv2.1 currents and tyrosine phosphorylation in transfected HEK 293 cells. In all, PTP ϵ antagonizes activation of Kv channels by tyrosine kinases *in vivo*, and affects Schwann cell function during a critical period of Schwann cell growth and myelination.

Keywords: myelination/potassium channel/Schwann cells/tyrosine kinase/tyrosine phosphatase

Introduction

Reversible phosphorylation of tyrosine residues in proteins plays a central role in regulation of cellular functions, and is a process controlled by the opposing actions of protein tyrosine kinases (PTKs) and tyrosine phosphatases (PTPases) (Hunter, 1995). Aberrant PTK activity has been linked repeatedly to a wide variety of human diseases, underscoring the pivotal role of accurate tyrosine phosphorylation in physiological processes. PTPases, which are molecularly, biochemically and physiologically distinct from PTKs, have not been studied as extensively as PTKs, although their intimate link to phosphorylation events indicates that PTPases are highly relevant in this respect.

PTPases are a structurally diverse family of transmembranal and cytoplasmic enzymes, of which >70 members have been identified in organisms ranging from viruses to man (Tonks and Neel, 1996). Transmembranal PTPases typically contain two catalytic domains each and are believed to bind extracellular molecules and to participate in signal transduction events (Schaapveld *et al.*, 1997; Zondag and Moolenaar, 1997). Cytoplasmic PTPases

generally contain a single catalytic domain flanked by protein domains, which either regulate the catalytic activity of the molecule or target it to particular regions within the cell (Mauro and Dixon, 1994; Denu and Dixon, 1998). Depending on its context, PTPase activity can enhance or decrease the intensity of transduced signals and is physiologically significant (Tonks and Neel, 1996; Neel and Tonks, 1997; Fischer, 1999). Not surprisingly, several key members of this family have been implicated in control of growth, differentiation and malignant transformation (Tonks and Neel, 1996; Parsons, 1998; den Hertog, 1999).

Protein tyrosine phosphatase epsilon (PTP ϵ ; gene symbol *Ptpre*) is somewhat unique among PTPases in that the single PTP ϵ gene contains two distinct promoters, each of which gives rise to a unique protein product: a transmembranal, receptor-type protein (tm-PTP ϵ) and a second protein, which is predominantly cytoplasmic (cyt-PTP ϵ) (Krueger *et al.*, 1990; Elson and Leder, 1995a,b; Nakamura *et al.*, 1996; Tanuma *et al.*, 1999). Although most of their sequences—including their catalytic domains—are identical, tm- and cyt-PTP ϵ possess unique N-termini that determine their different subcellular localizations and probably distinct physiological roles (Elson and Leder, 1995b). Both forms of PTP ϵ bind Grb2 (Toledano-Katchalski and Elson, 1999); tm-PTP ϵ down-regulates insulin receptor signaling in transfected cells (Moller *et al.*, 1995), has been linked with promotion of mammary tumorigenesis *in vivo* (Elson and Leder, 1995a; Elson, 1999) and may play a role in regulating osteoclast function (Schmidt *et al.*, 1996).

It is reasonable to expect that additional physiological roles of PTP ϵ will involve molecules whose activity is regulated by tyrosine phosphorylation. Prominent among these are K⁺ channels, as several studies have shown that these are substrates of protein kinase activities (Levitan, 1994, 1999; Siegelbaum, 1994; Jonas and Kaczmarek, 1996). Heterologous expression studies indicate that the activity of several members of the Kv1 family of delayed-rectifier K⁺ (Kv) channels is altered following phosphorylation by non-receptor and receptor tyrosine kinases (Huang *et al.*, 1993; Timpe and Fantl, 1994; Holmes *et al.*, 1996; Bowlby *et al.*, 1997; Fadool *et al.*, 1997; Tsai *et al.*, 1997; Wang, 1999). *In vivo*, phosphorylation of K⁺ channels by PTKs can either activate or down-regulate channel activity. Along these lines, activated insulin receptor increases delayed-rectifier K⁺ currents in *Aplysia* bag cell neurons (Jonas *et al.*, 1996), while in the Jurkat human T-cell line, p56^{lck}-mediated phosphorylation of the Kv1.3 potassium channels down-regulates voltage-sensitive K⁺ currents (Szabo *et al.*, 1996). Recently, we showed that the delayed-rectifier *I_K* is markedly up-regulated following intracellular application of recombinant Fyn tyrosine kinase to mouse Schwann

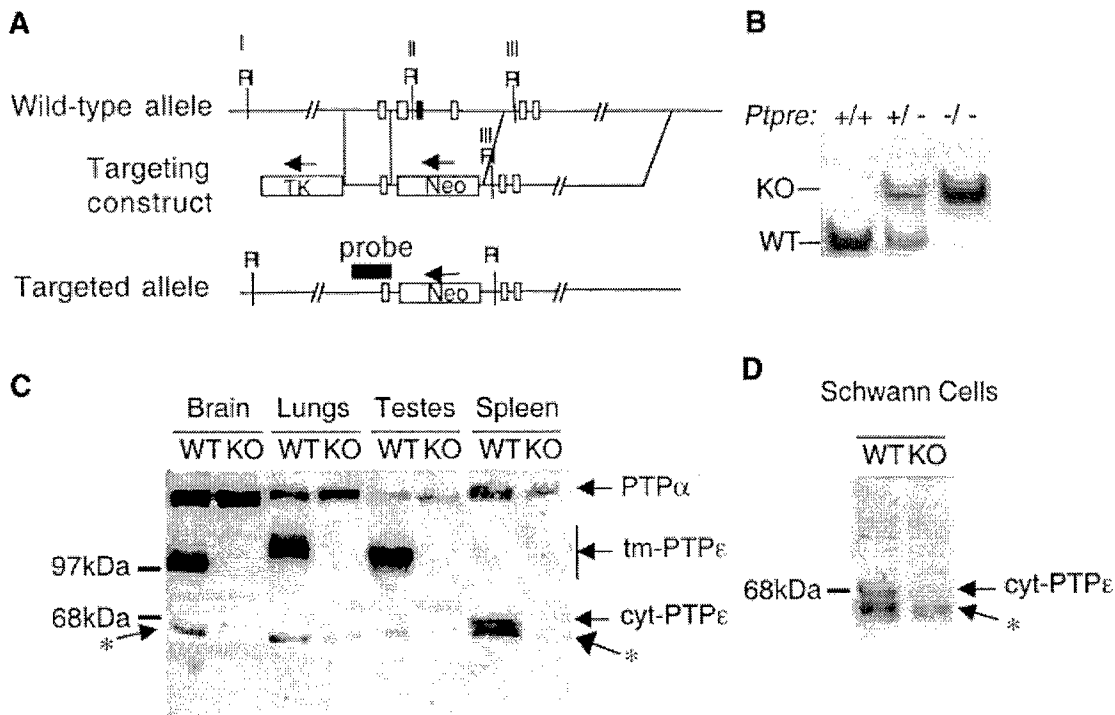


Fig. 1. Generation and characterization of *Ptpre*^{-/-} mice. (A) Schematic representations of the region of the mouse *Ptpre* gene chosen for targeting (top), targeting construct (middle) and recombinant allele (bottom). Homologous recombination removed three exons (rectangles), including one (black rectangle) containing C277, the catalytic cysteine of the membrane-proximal catalytic domain. Flanking regions are 1.2 (5') and 10 kb (3') in length. RI-EcoRI sites in the targeted region. (B) DNA blot analysis of genomic DNA from *Ptpre*^{+/+}, *Ptpre*^{+/-} and *Ptpre*^{-/-} mice. Genomic DNA was digested with *Eco*RI and probed with the genomic fragment indicated in (A). Fragments originating in the wild-type (WT; 10.0 kb) and targeted (KO; 11.8 kb) alleles, respectively, are indicated. (C) Protein blot documenting lack of expression of PTPε in *Ptpre*^{-/-} mice. Protein extracts from the indicated organs of WT or KO mice were analyzed using an anti-PTPε antibody (Elson and Leder, 1995a). Antibody also cross-reacts with PTPα as indicated in the figure. Variations in size of tm-PTPε from brain, lungs and testes are due to tissue-specific glycosylation (Elson and Leder, 1995a). Note the presence in several lanes of PTPε-related bands migrating at ~68 kDa (asterisk), slightly faster than cyt-PTPε. (D) Protein blot analysis as in (C), documenting expression levels of cyt-PTPε in primary WT or KO Schwann cells derived from 3- to 5-day-old pups.

cells, and is down-regulated upon exposure to tyrosine kinase inhibitors (Sobko *et al.*, 1998a; Peretz *et al.*, 1999). In contrast, very little is known about the *in vivo* regulation of K⁺ channel activity by PTPases, although a recent study showed that *in vitro* expression of PTPα correlates with activation of Kv1.2 channels (Tsai *et al.*, 1999).

The intimate connection between regulation of Kv channels and PTKs prompted us to examine whether PTPε is involved in this process *in vivo*. For this purpose we examined Kv channel function in gene-targeted mice that lack PTPε (*Ptpre*^{-/-} mice). Lack of PTPε causes hypomyelination of sciatic nerve axons in newborn mice, together with increased Kv channel activity and hyperphosphorylation of the Kv1.5 and Kv2.1 α-subunits in cultured primary Schwann cells and sciatic nerve tissue. *In vitro* studies show that Kv2.1 interacts with the active site of PTPε, and that active PTPε can down-regulate Kv1.5 and Kv2.1 channel activity and phosphorylation. Our results indicate that a unique role of PTPε *in vivo* is to down-regulate Kv channel activity in post-natal mice, thereby antagonizing channel activation by tyrosine kinases such as Src and Fyn. PTPε is then part of the finely tuned molecular mechanism, which regulates Kv channel activity during a critical period of Schwann cell development and myelination of peripheral nerves.

Results

PTPε-deficient mice exhibit peripheral myelination abnormalities

The *Ptpre* gene was targeted by replacing the genomic sequence corresponding to amino acid residues 262–411 of mature tm-PTPε with a selectable neomycin resistance gene (Figure 1A). This region is common to both tm- and cyt-PTPε (Elson and Leder, 1995b), and was chosen so as to disrupt both forms of the enzyme. Following electroporation and selection, clones of *Ptpre*^{+/+} embryonic stem (ES) cells were used to generate chimeric mice and subsequently *Ptpre*^{+/-} and *Ptpre*^{-/-} mice (Figure 1B). tm-PTPε is known to be expressed mainly in brain, as well as in lungs and testes, while cyt-PTPε is expressed mainly in the hematopoietic system (Elson and Leder, 1995b; Mukouyama *et al.*, 1997); protein blot analysis indicated that *Ptpre*^{-/-} mice do not express tm- or cyt-PTPε proteins in these tissues (Figure 1C). As the antibody used was raised against a peptide located upstream of the targeting site (Elson and Leder, 1995a), it would have allowed the detection of possible truncated protein products; none were detected. The antibody cross-reacts with the closely related PTPα; no compensatory changes in the amounts of PTPα protein were detected in the organs examined (Figure 1C).

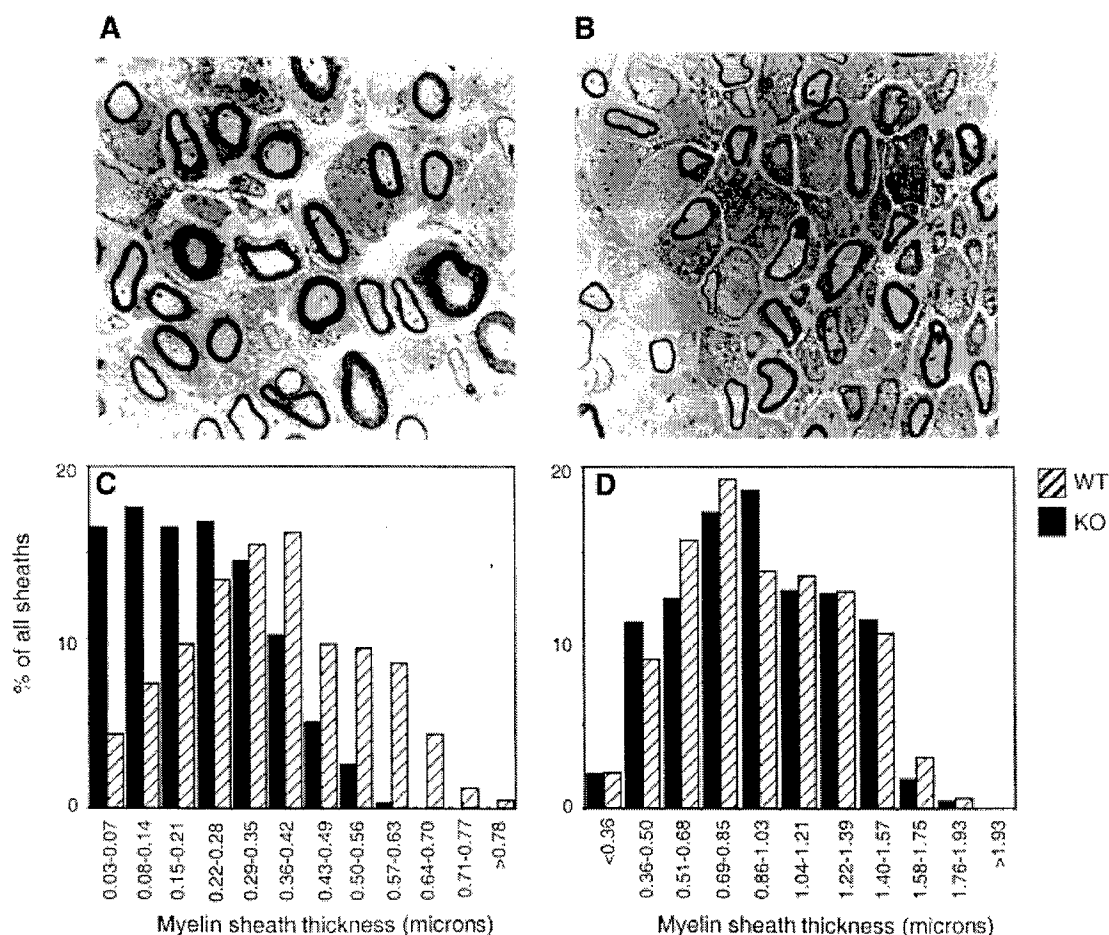


Fig. 2. Reduced myelination of axons in sciatic nerves of 5-day-old *Ptpre*^{-/-} mice. Cross-section of sciatic nerve of WT (A) or *Ptpre*^{-/-} (B) mice. Magnification 3400 \times . (C) Distribution of widths of myelin sheaths of axons in sciatic nerves of 5-day-old WT (hatched bars) and KO (black bars) mice. Mean sheath thicknesses (in μ m, \pm SEM) were: *Ptpre*^{-/-}: 0.23 ± 0.05 , $n = 456$; wild type: 0.37 ± 0.06 , $n = 526$ ($p < 0.0001$ by the Mann-Whitney test). (D) As in (C), data from adult, 8-month-old male mice. Mean sheath thicknesses (in μ m, \pm SEM) were: *Ptpre*^{-/-}: 0.92 ± 0.02 , $n = 448$; wild type: 0.94 ± 0.02 , $n = 331$.

Ptpre^{-/-} mice were born at the expected Mendelian ratios from matings of *Ptpre*^{+/-} mice and were normal in appearance. However, detailed examination of early post-natal (3- to 5-day-old) *Ptpre*^{-/-} mice revealed myelination defects in the peripheral nervous system. Specifically, myelin sheaths surrounding axons in sciatic nerve fibers of *Ptpre*^{-/-} mice were significantly thinner than in WT (wild-type *Ptpre*^{+/-}) mice (Figure 2A and C). Thinning of myelin sheaths was most evident in the nearly total disappearance of the very thickest sheaths ($>0.6 \mu$ m) from *Ptpre*^{-/-} sciatic nerves, together with a 2.6-fold increase in the fraction of thinly myelinated axons ($<0.14 \mu$ m). Interestingly, myelination of sciatic nerve axons in adult (8 months) *Ptpre*^{-/-} mice was normal (Figure 2D). These results suggest that PTPe performs a unique role in Schwann cells during early myelinogenesis, an important developmental period when Schwann cells cease dividing and differentiate. In adult mice, on the other hand, this function is either not required or can be performed by other PTPases.

Up-regulation of Kv channel activity and phosphorylation in PTPe-deficient Schwann cells

Recent evidence indicates that phosphorylation of Kv channels by Src family kinases could be important for Schwann cell development and peripheral myelinogenesis. In particular, the Fyn tyrosine kinase constitutively activates Kv channels in proliferating Schwann cells (Sobko et al., 1998a; Peretz et al., 1999). After birth, a developmental decrease in Fyn kinase and Kv channel activities may contribute to the exit of Schwann cells from the cell cycle and onset of myelination (Sobko et al., 1998a,b). As tyrosine phosphatases generically counter activities of tyrosine kinases, we sought to determine whether lack of PTPe could modulate Kv channels and Schwann cell function *in vivo*. WT Schwann cells express cyt-PTPe; the enzyme is missing from cells of *Ptpre*^{-/-} mice (Figure 1D). The activity of Kv channels was monitored by recording K⁺ currents in Schwann cells from WT and *Ptpre*^{-/-} mice using the whole-cell configuration

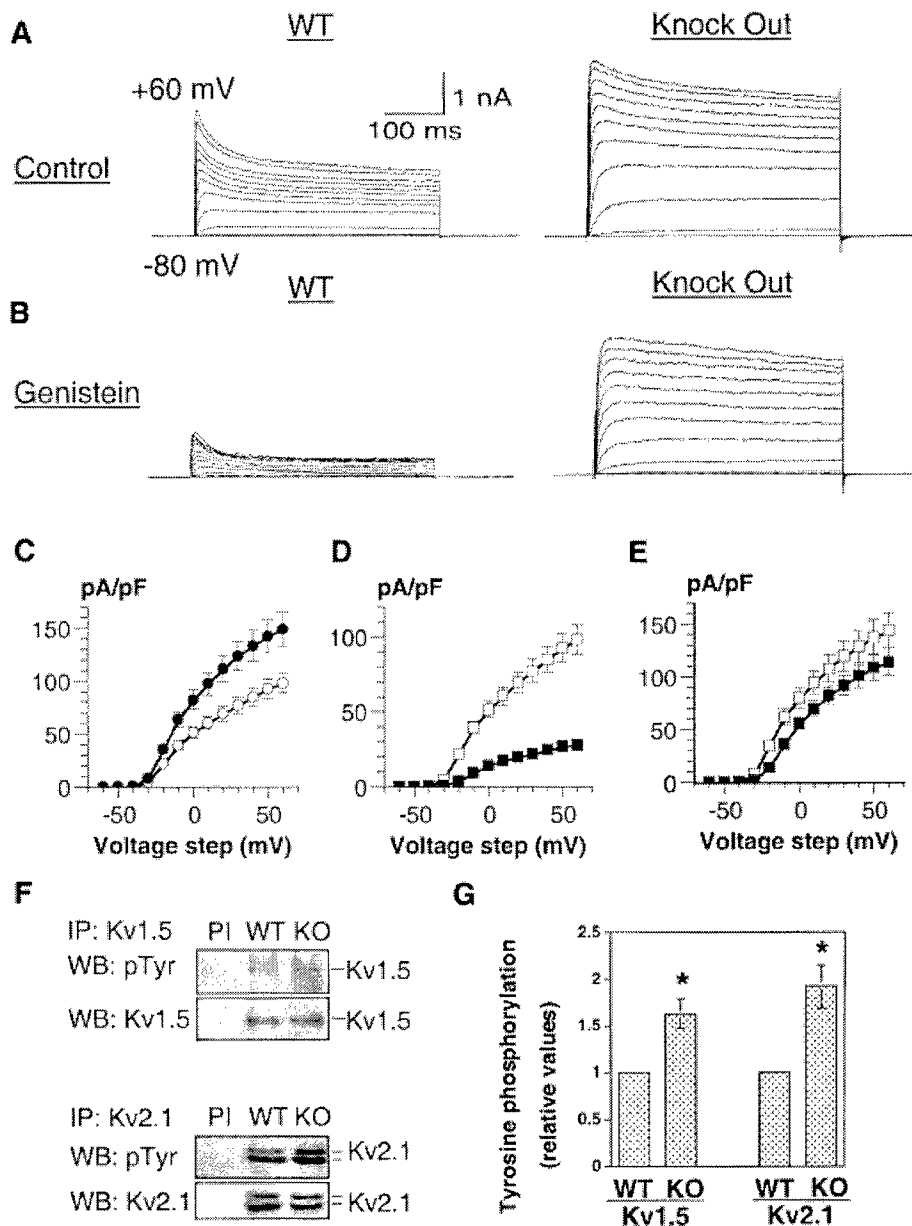


Fig. 3. Characteristics of voltage-gated K⁺ currents in Schwann cells from WT and *Ptpre*^{-/-} mice and tyrosine phosphorylation of delayed-rectifier Kv channel α -subunits. (A and B) Whole-cell K⁺ currents recorded from WT and *Ptpre*^{-/-} Schwann cells, before (A) and after (B) application of 100 μ M genistein for 20 min. Cells were stepped from a holding potential of -80 mV to +60 mV in +10 mV increments for 400 ms pulse duration. (C) The K⁺ current density (pA/pF) of WT ($n = 35$, open circles) and *Ptpre*^{-/-} ($n = 40$, solid circles) Schwann cells was plotted against voltage steps (mV). *Ptpre*^{-/-} Schwann cells exhibit a significantly higher current density when compared with WT cells ($p < 0.01$). (D and E) Current density-voltage relationships ($n = 28$) of WT (D) and *Ptpre*^{-/-} (E) Schwann cells, before (open squares) and following (solid squares) exposure to 100 μ M genistein for 20 min. (F) Representative experiment showing hyperphosphorylation of Kv1.5 and Kv2.1 in Schwann cells of *Ptpre*^{-/-} mice as compared with WT cells. Total protein was immunoprecipitated with anti-Kv1.5 or anti-Kv2.1 antibodies followed by blotting and probing with anti-phosphotyrosine antibodies. Blots were then stripped and re-probed for Kv1.5 or Kv2.1; similar results were obtained for Kv2.1 in sciatic nerve tissue (not shown). (G) Quantification of tyrosine phosphorylation of Kv1.5 and Kv2.1 in WT and *Ptpre*^{-/-} Schwann cells. Phosphorylation of Kv1.5 is increased by 62.5 \pm 15.4% ($n = 4$), while that of Kv2.1 is increased by 91.3 \pm 22.8% ($n = 6$); an asterisk indicates statistical significance ($p < 0.01$) in both cases.

of the patch-clamp technique. These measurements revealed that the maximal K⁺ current density of *Ptpre*^{-/-} Schwann cells increased significantly by 52% when compared with WT cells [from 98.02 \pm 9.86 pA/pF (WT) to 149.13 \pm 16.11 pA/pF (*Ptpre*^{-/-}) at +60 mV; Figure 3A and C]. No difference was found in the voltage-dependence characteristics of channel activation between

Ptpre^{-/-} and WT Schwann cells (data not shown), suggesting that PTP ϵ could affect the number of functional channels or the unitary channel conductance. In parallel, two Kv α -subunits, Kv1.5 and Kv2.1, were significantly hyperphosphorylated in *Ptpre*^{-/-} Schwann cells (Figure 3F and G) and in sciatic nerve tissue (data not shown). In *Ptpre*^{-/-} Schwann cells, the Kv1.5 and Kv2.1 phospho-

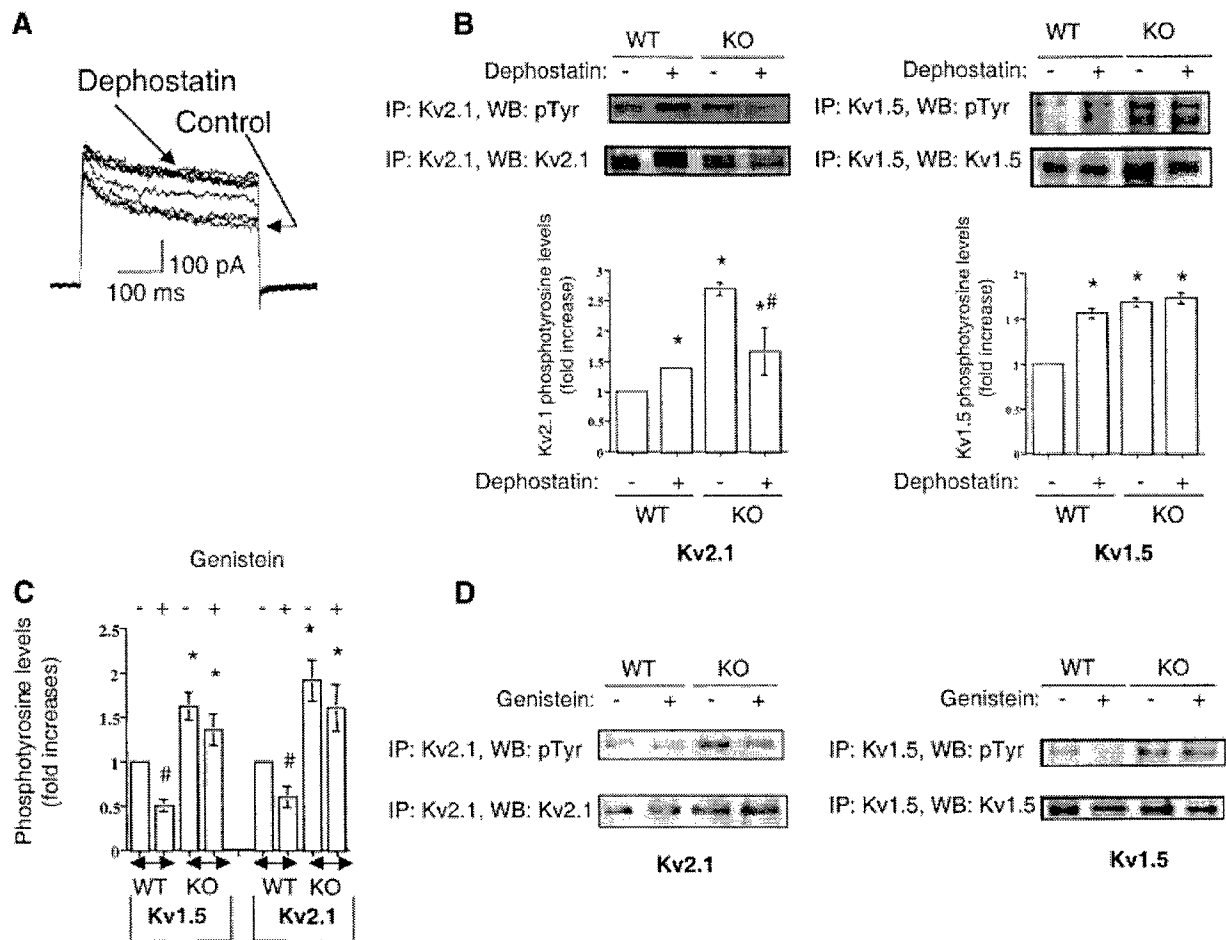


Fig. 4. Effects of dephostatin and genistein on tyrosine phosphorylation of Kv1.5 and Kv2.1 channel α-subunits. (A) Train of current traces recorded from the same WT *Ptpre*^{+/+} Schwann cell, before and after 50 μM dephostatin treatment (20 min). The cell was stepped every minute from a holding potential of -80 mV to +40 mV (400 ms). (B) Effects of dephostatin (50 μM, 20 min) on tyrosine phosphorylation of Kv1.5 and Kv2.1 α-subunits in WT *Ptpre*^{+/+} and KO *Ptpre*^{-/-} Schwann cells. Upper panel, representative experiment showing that dephostatin produces an increase in tyrosine phosphorylation of Kv1.5 and Kv2.1 channel α-subunits in WT but not in KO Schwann cells. Lower panel, quantification of tyrosine phosphorylation of Kv1.5 and Kv2.1 in WT and KO Schwann cells before and after dephostatin treatment. Dephostatin increases Kv1.5 and Kv2.1 tyrosine phosphorylation by 56 ± 6% and 39 ± 2%, respectively (*n* = 3, *p* < 0.05). An asterisk indicates a statistically higher value when compared with WT untreated Schwann cells (*p* < 0.05). Dephostatin significantly reduces, by 38 ± 14%, the phosphotyrosine levels of Kv2.1 in KO Schwann cells (*n* = 3, #*p* < 0.05). Total proteins were immunoprecipitated, blotted and probed as in Figure 3F. (C) Quantification of the effects of genistein (100 μM, 20 min) on tyrosine phosphorylation of Kv1.5 and Kv2.1 in WT and KO Schwann cells. Genistein reduces by 50 ± 7% and 40 ± 13% the tyrosine phosphorylation levels of Kv1.5 and Kv2.1, respectively (*n* = 3, #*p* < 0.05). An asterisk indicates a statistically higher value when compared with WT untreated Schwann cells (*p* < 0.05). (D) Representative experiment showing that genistein decreases significantly the tyrosine phosphorylation of Kv1.5 and Kv2.1 channel α-subunits in WT. The inhibitory effect of genistein is much weaker, if present at all, in KO Schwann cells. Total proteins were immunoprecipitated, blotted and probed as in Figure 3F.

tyrosine levels were up-regulated by 62.5 ± 15.4% (*n* = 4, *p* < 0.01) and 91.3 ± 22.8% (*n* = 6, *p* < 0.01), respectively, when compared with WT cells (Figure 3F and G). The data indicate that cyt-PTPase activity normally leads to dephosphorylation of Kv α-subunits and to down-regulation of Kv channel activity *in vivo*. The data also support the correlation observed between hyperphosphorylation of Kv channel α-subunits and increased channel activity (Sobko et al., 1998a; Peretz et al., 1999).

Up-regulation of Kv currents observed in *Ptpre*^{-/-} Schwann cells agrees with results obtained in WT Schwann cells following broad-spectrum inhibition of tyrosine phosphatases or tyrosine kinases. Dephostatin, an inhibitor of tyrosine phosphatases, causes up-regulation of

Kv currents and hyperphosphorylation of Kv1.5 and Kv2.1 channel α-subunits in WT Schwann cells (Figure 4A and B). Interestingly, dephostatin-mediated up-regulation of Kv1.5 and Kv2.1 tyrosine phosphorylation is lost in *Ptpre*^{-/-} Schwann cells (Figure 4A and B). Dephostatin can eventually reduce the phosphotyrosine levels of Kv2.1 in cells from knockout animals, probably by inhibiting other tyrosine phosphatases, which modulate Kv2.1 channels in a manner different from that of cyt-PTPase (Figure 4B). In agreement with this result, the tyrosine kinase inhibitor genistein exerts the opposite effect and down-regulates Kv channel activity in WT Schwann cells (Sobko et al., 1998a; Peretz et al., 1999). Thus, in WT cells, genistein down-regulates tyrosine phosphorylation levels of Kv1.5

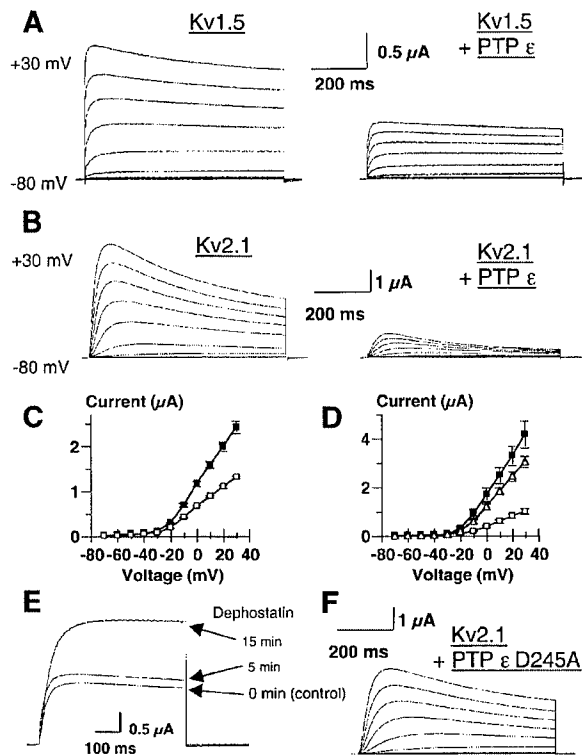


Fig. 5. Effects of cyt-PTPε and D245A cyt-PTPε substrate-trapping mutant on Kv2.1 and Kv1.5 K⁺ currents expressed in *Xenopus* oocytes. (A) Macroscopic K⁺ currents recorded from oocytes microinjected with Kv1.5 (left) or Kv1.5 plus cyt-PTPε cRNA (right) were elicited by depolarizing pulses (700 ms) from a -80 mV holding potential to +30 mV in 10 mV increments. (B) Macroscopic K⁺ currents recorded as in (A), from oocytes microinjected with Kv2.1 (left) or Kv2.1 plus cyt-PTPε cRNAs (right). (C) Current-voltage relationships of Kv1.5 ($n = 14$, solid squares) and Kv1.5 plus cyt-PTPε-mediated K⁺ currents ($n = 16$, open squares). cyt-PTPε decreases Kv1.5 currents significantly ($p < 0.01$). (D) Current-voltage relationships of Kv2.1 ($n = 15$, solid squares), Kv2.1 plus cyt-PTPε ($n = 16$, open squares) and Kv2.1 plus D245A PTPε-mediated K⁺ currents ($n = 14$, open triangles). cyt-PTPε decreases Kv2.1 currents significantly ($p < 0.01$). (E) Representative train of current traces recorded from oocytes microinjected with Kv2.1 and cyt-PTPε cRNAs, before and after 50 μM dephostatin treatment. The oocyte was stepped from a holding potential of -80 mV to +20 mV (600 ms). (F) Macroscopic K⁺ currents recorded as in (A), from oocytes microinjected with Kv2.1 and the D245A PTPε mutant.

and Kv2.1 channel α -subunits by $50 \pm 7\%$ ($n = 3$, $p < 0.01$) and $40 \pm 12\%$ ($n = 3$, $p < 0.05$), respectively (Figure 4C and D). Tyrosine phosphorylation of Kv channel α -subunits is then finely regulated by opposing activities of tyrosine kinases and tyrosine phosphatases (Jonas and Kaczmarek, 1996; Levitan, 1999). These experiments also link cyt-PTPε in particular to down-regulation of Kv currents, as the effects of genistein and dephostatin are markedly altered in *Ptpre*^{-/-} Schwann cells. Genistein (100 μM) reduces Kv current density by 62% in WT Schwann cells, compared with only a 27% reduction in *Ptpre*^{-/-} cells (Figure 3A, B, D and E). In line with this result, genistein does not alter significantly the Kv1.5 or Kv2.1 phosphotyrosine steady-state levels in *Ptpre*^{-/-} Schwann cells (Figure 4C and D). Similar results were obtained with herbimycin A (data not shown). The inhibitory effect of genistein or herbimycin A on Kv

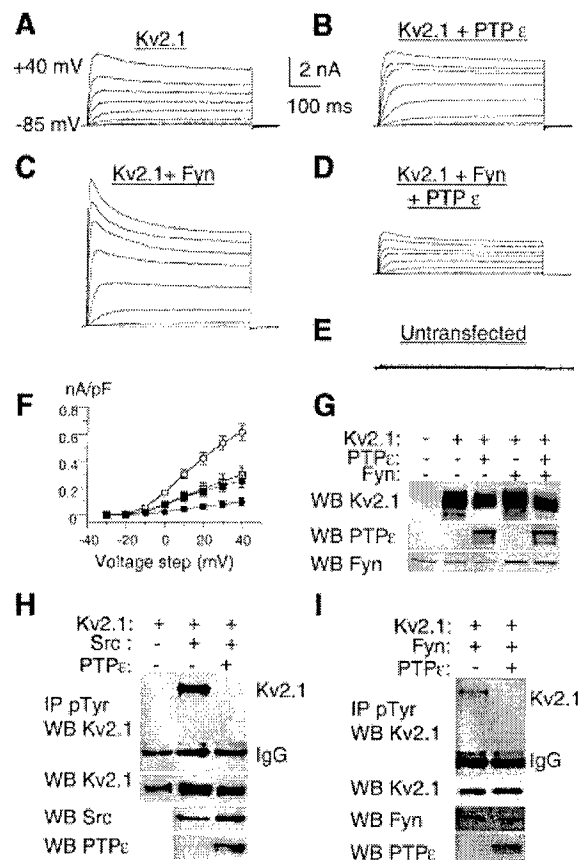


Fig. 6. Effects of cyt-PTPε, activated Fyn and Src kinases on Kv2.1 tyrosine phosphorylation and K⁺ currents expressed in HEK 293 cells. (A-E) Representative whole-cell K⁺ currents from cells transfected with Kv2.1 (A), Kv2.1 + cyt-PTPε (B), Kv2.1 + activated Fyn kinase (C), Kv2.1 + activated Fyn kinase + cyt-PTPε (D) cDNAs, respectively, as well as from an untransfected cell (E). Currents were elicited by depolarizing pulses (500 ms) from a -85 mV holding potential to +40 mV in 20 mV increments. The scale bars are the same for A-E. (F) Current-voltage relationships of K⁺ currents produced by cells transfected with Kv2.1 ($n = 15$, open squares), Kv2.1 + cyt-PTPε ($n = 5$, solid squares), Kv2.1 + activated Fyn kinase ($n = 12$, open circles) and Kv2.1 + activated Fyn kinase + cyt-PTPε ($n = 14$, filled circles) cDNAs. (G) Documentation of expression of Kv2.1, cytPTPε and Fyn in HEK 293 cells used for electrophysiological studies presented in (A-E). Cells were transfected with plasmids for the indicated proteins, and protein blots prepared from their extracts were probed with the indicated antibodies. (H and I) Cyt-PTPε reduces, respectively, Src- (H) and Fyn-mediated (I) phosphorylation of Kv2.1 in transfected cells. HEK 293 cells were transiently transfected with Kv2.1, activated Fyn (Y531F), activated Src (Y527F), cyt-PTPε cDNAs, or combinations thereof. Tyrosine-phosphorylated proteins were immunoprecipitated and the amount of Kv2.1 in the precipitate was analyzed by protein blotting (top panel). The second, third and fourth panels document expression of Kv2.1, Fyn/Src and cyt-PTPε, respectively, in the transfected cells.

channel activity is then partly countered by lack of cyt-PTPε activity in *Ptpre*^{-/-} Schwann cells.

cyt-PTPε down-regulates Kv channel activity and phosphorylation in vitro

To investigate direct modulation of Kv channels by cyt-PTPε further, we co-expressed Kv1.5 or Kv2.1 with cyt-PTPε in *Xenopus* oocytes and HEK 293 cells (Figures 5 and 6). In *Xenopus* oocytes, co-expression of Kv1.5 or

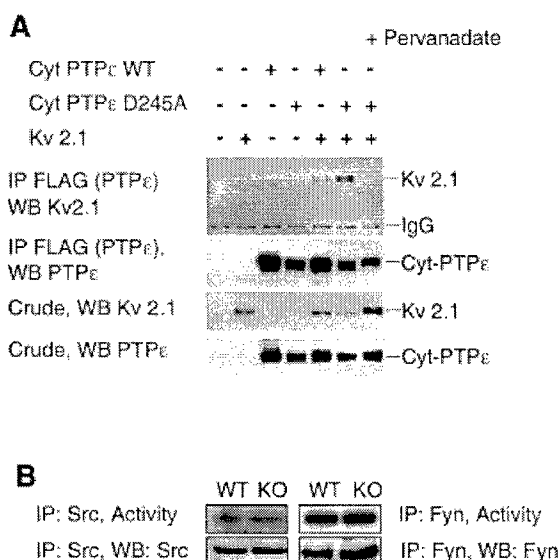


Fig. 7. (A) Kv2.1 preferentially associates with the D245A cyt-PTP ϵ substrate-trapping mutant. HEK 293 cells were transiently transfected with wild-type cyt-PTP ϵ , D245A cyt-PTP ϵ , Kv2.1, or combinations thereof. Following lysis, PTP ϵ was immunoprecipitated via its attached FLAG tag; associated Kv2.1 was detected by protein blot analysis of immunoprecipitates (top panel). The second, third and fourth panels document expression of cyt-PTP ϵ and Kv2.1 and immunoprecipitation of cyt-PTP ϵ in the various transfections. The presence of pervanadate (right-most lane) somewhat reduces the electrophoretic mobility of some of the proteins examined, most likely by more effectively reducing their dephosphorylation by phosphatases present in cell lysates during precipitation. (B) Expression and activities of Src and Fyn are unaltered in *Ptpre*^{-/-} Schwann cells. Total protein from primary Schwann cells of 3- to 5-day-old mice were immunoprecipitated with anti-Src or anti-Fyn antibodies. A fraction of the precipitated kinases was allowed to phosphorylate acid-denatured enolase *in vitro* in the presence of [γ -³²P]ATP, followed by SDS-PAGE analysis and exposure to film (top panels). Another fraction of the immunoprecipitated material was analyzed by protein blotting for expression levels of Src or Fyn (bottom panels). Tyrosine phosphorylation levels of both kinases were unaltered (not shown). The experiment shown is representative of three performed.

Kv2.1 with cyt-PTP ϵ leads to a marked decrease in Kv current amplitudes, with 46 and 76% inhibition, respectively (Figure 5A–D). Similar to conclusions from our primary Schwann cell experiments, the inhibitory action of PTP ϵ does not involve significant changes in the kinetics or in the voltage dependence of channel activation (Figure 5A and B). PTP ϵ catalytic activity is important for this process, as a catalytically inactive, substrate-trapping mutant (Flint *et al.*, 1997) of cyt-PTP ϵ (D245A cyt-PTP ϵ) inhibits only 27% of Kv current amplitude, compared with 76% inhibition by wild-type cyt-PTP ϵ (Figure 5D and F). Residual inhibition by D245A cyt-PTP ϵ may result from binding of the inactive phosphatase to Kv2.1 (see below) in a manner which, though not dephosphorylating Kv2.1, may nonetheless impede its activity. These data are in line with those obtained with *Ptpre*^{-/-} mice and indicate that cyt-PTP ϵ can down-regulate activity of Kv channels by reducing tyrosine phosphorylation of their α -subunits. Consistent with these results, the tyrosine phosphatase inhibitor dephostatin causes up-regulation of K⁺ currents produced by co-expression Kv2.1 and cyt-PTP ϵ in *Xenopus* oocytes

(Figure 5E). In HEK 293 cells, cyt-PTP ϵ does not depress the basal Kv2.1 current activity (Figure 6A, B and F). This feature differs from *Xenopus* oocyte expression and is probably due to the lack of basal tyrosine phosphorylation of Kv2.1 channel α -subunits in HEK 293 cells, as revealed by our immunoprecipitation experiments (Figure 6H). However, when tyrosine phosphorylation of Kv2.1 α -subunits is stimulated by co-expressing activated Fyn kinase (Y531F), the Kv2.1 K⁺ current amplitude is up-regulated by >2-fold (617 ± 58 pA/pF, $n = 12$, $p < 0.01$) when compared with control Kv2.1-expressing cells (298 ± 63 pA/pF, $n = 15$) (Figure 6A, C, F and I). Under these stimulatory conditions, co-expression of cyt-PTP ϵ leads to a profound down-regulation of Kv2.1 current amplitude (101 ± 28 pA/pF, $n = 14$, $p < 0.001$). cyt-PTP ϵ totally suppresses the Fyn kinase stimulation of Kv2.1 current and tyrosine phosphorylation (Figure 6C, D, F and I). Similar results were obtained when Kv2.1 tyrosine phosphorylation was stimulated by activated Src kinase (Figure 6H). Correlative western blots show that co-expression of either activated Fyn kinase, activated Src kinase or cyt-PTP ϵ does not alter significantly the expression levels of Kv2.1 channel α -subunits (Figure 6G–I; see also Figure 7A).

Kv2.1 binds the active site of cyt-PTP ϵ

The above results suggest that cyt-PTP ϵ binds and dephosphorylates α -subunits of Kv channels. In agreement with this model, several-fold more Kv2.1 co-precipitate with a D245A substrate-trapping mutant (Flint *et al.*, 1997) of cyt-PTP ϵ than with wild-type cyt-PTP ϵ (Figure 7A). Significantly, enhanced binding to D245A cyt-PTP ϵ does not occur in the presence of sodium pervanadate (Figure 7A), which irreversibly oxidizes the conserved cysteine residue located at the active site of tyrosine phosphatases (Huyer *et al.*, 1997). Oxidation of this cysteine residue by pervanadate is known to disrupt active site-mediated binding of D-to-A type phosphatase mutants to their putative substrates (Flint *et al.*, 1997; Huyer *et al.*, 1997). These results indicate that Kv2.1 interacts mainly with the active site of cyt-PTP ϵ , consistent with Kv2.1 being a substrate of cyt-PTP ϵ . An alternative model based on findings in PTP α -deficient mice (Ponniah *et al.*, 1999; Su *et al.*, 1999) suggests that cyt-PTP ϵ could indirectly control phosphorylation of Kv channel α -subunits by regulating activities of Src family tyrosine kinases. Fyn is the major Src-family tyrosine kinase active towards Kv channels in Schwann cells (Sobko *et al.*, 1998a); yet, expression, activity and overall tyrosine phosphorylation of both Fyn and Src are unchanged in *Ptpre*^{-/-} Schwann cells (Figure 7B and data not shown). Although this result does not rule out interactions between Fyn/Src and cyt-PTP ϵ , it appears that these kinases are not the major mediators of cyt-PTP ϵ activity towards Kv channels in the system studied here.

Discussion

Results presented here indicate that lack of cyt-PTP ϵ expression results in reduced myelination of sciatic nerve axons in early post-natal *Ptpre*^{-/-} mice. This finding parallels increased activity of voltage-gated potassium channels in primary Schwann cells derived from these

mice, which in turn is mediated by hyperphosphorylation of Kv channel α -subunits. *Ptpre*^{-/-} Schwann cells modulate phosphorylation of Kv1.5 and Kv2.1 differently than wild-type cells in response to broad-spectrum inhibition of tyrosine phosphatases or kinases, further attesting to the importance of this PTPase in early post-natal Schwann cells. The ability of cyt-PTP ϵ to reduce phosphorylation and to down-regulate Kv1.5 and Kv2.1 can be reproduced in heterologous expression systems. Taken together, these findings indicate that cyt-PTP ϵ dephosphorylates α -subunits of delayed-rectifier Kv channels, and that lack of cyt-PTP ϵ most likely causes Schwann cell dysfunction *in vivo* in young mice. This study provides the first *in vivo* evidence of a functional link between a specific tyrosine phosphatase and regulation of Kv channel activity, and clearly defines its physiological consequences.

Although the above data suggest that cyt-PTP ϵ plays a significant role in regulation of Kv phosphorylation in Schwann cells, the fact that myelination of sciatic nerve axons is normal in adult *Ptpre*^{-/-} mice indicates that other PTPases participate in this process as well. The need for cyt-PTP ϵ for proper Schwann cell function then likely decreases as mice age. A possible candidate for compensating lack of cyt-PTP ϵ in Schwann cells is PTP σ , as young mice lacking PTP σ exhibit reduced myelination of sciatic nerve axons caused by a yet undetermined molecular mechanism (Wallace *et al.*, 1999; M.Tremblay, personal communication). Nonetheless, the existence of myelination defects in post-natal *Ptpre*^{-/-} mice demonstrates that cyt-PTP ϵ performs a unique function in Schwann cells of such mice, and that other phosphatases cannot replace this enzyme at this developmental stage.

Recent data have shown that K⁺ channel activity can be either up-regulated or down-regulated by PTKs depending on the physiological context. Heterologous expression studies indicate that the K⁺ channel activity of several Kv1 family members is down-regulated by tyrosine kinases. In HEK 293 cells, phosphorylation of Kv1.3 by activated v-src or by epidermal growth factor treatment leads to a decrease in current amplitude (Holmes *et al.*, 1996; Bowlby *et al.*, 1997; Fadool *et al.*, 1997). The current amplitudes of several other Kv channels, such as Kv1.2 and Kv1.5, are known to be strongly suppressed by tyrosine kinases (Timpe and Fantl, 1994; Holmes *et al.*, 1997; Tsai *et al.*, 1997). However, a recent *in vitro* study demonstrates that tyrosine phosphorylation can up-regulate the activity of the Kv1.1 channel (Wang *et al.*, 1999). Differential regulation of K⁺ channel activity by tyrosine phosphorylation has also been observed *in vivo*. Activation of the insulin receptor inhibits Kv current amplitude in olfactory bulb neurons (Fadool and Levitan, 1998). On the other hand, positive modulation of transient K⁺ channels by PTKs was described in rat ventricular cardiac cells (Guo *et al.*, 1995). Ca²⁺-activated K⁺ channels are up-regulated by PTK activation in mouse fibroblasts and in Chinese hamster ovary cells (Prevarskaya *et al.*, 1995; Decker *et al.*, 1998). More recently, ceramide was found to increase the delayed-rectifier K⁺ current via PTK activation in cultured cortical neurons, and this process parallels increased apoptosis of these cells (Yu *et al.*, 1999). Similarly, our recent work showed that the Fyn

tyrosine kinase increases delayed-rectifier K⁺ channel activity in mouse Schwann cells, and that exposure to tyrosine kinase inhibitors markedly down-regulates Kv current amplitude and inhibits cell proliferation in this system (Sobko *et al.*, 1998a,b).

The intimate connections between activities of PTKs and PTPases argue that dephosphorylation of Kv channels may also affect channel activity in a manner dependent upon the circumstances of the reaction. Interestingly, *in vitro* expression of PTP α , a phosphatase closely related to the transmembrane, receptor-like form of PTP ϵ , has been shown to correlate with activation of Kv1.2 (Tsai *et al.*, 1999), the opposite of the effect that cyt-PTP ϵ has on Kv1.5 and Kv2.1. This result may reflect basic differences in the manner by which PTP ϵ and PTP α function, or in the effect dephosphorylation has on different Kv α -subunits. Alternatively, as our results are based on both *in vivo* (Schwann cells) and heterologous *in vitro* (*Xenopus* oocyte, HEK 293 cells) studies, this discrepancy may reflect differences in the molecular backgrounds of native versus heterologous cells and their influence on PTP α activity.

Our findings indicate that Kv channel phosphorylation in Schwann cells is regulated by opposing activities of tyrosine kinases and tyrosine phosphatases. One could then expect that inhibition of tyrosine kinases by genistein would offset Kv channel activation normally observed in *Ptpre*^{-/-} Schwann cells. This effect is indeed observed upon treatment of *Ptpre*^{-/-} Schwann cells with genistein; however, the magnitude of the effect is very mild. This may reflect residual kinase activity, which is not inhibited by genistein and which is now detectable in the absence of PTP ϵ . Alternatively, blockade of tyrosine kinases by genistein may be less effective in the context of defective regulation of tyrosine dephosphorylation, which exists in *Ptpre*^{-/-} Schwann cells.

The precise link between Kv channel activity and Schwann cell-mediated myelination is unclear at the present time. However, there is temporal correlation between decreases in activities of Src-related tyrosine kinases and of Kv channels, on the one hand, and exit of Schwann cells from the cell cycle and onset of myelination, on the other hand (Sobko *et al.*, 1998a,b; MacFarlane and Sontheimer, 2000). Our data indicate that lack of PTP ϵ results in increased tyrosine phosphorylation and activities of Kv channels, possibly delaying exit of *Ptpre*^{-/-} Schwann cells from the cell cycle and resulting in delayed myelination.

In all, results presented here indicate that, together with the Src family tyrosine kinases, cyt-PTP ϵ is part of the finely tuned molecular mechanism that regulates Kv channel activity during Schwann cell development and myelination of peripheral nerves *in vivo*. Schwann cell dysfunction and myelination defects are known to be associated with severe human neurological diseases. Better understanding of how Schwann cell functions are regulated at the molecular level could increase the chances of eventually controlling such diseases.

Materials and methods

Gene targeting and genotyping

Two recombinant phage-containing fragments of mouse genomic DNA from the *Ptpre* locus were isolated from a strain 129 mouse genomic

library (Stratagene) using a fragment of the mouse *tm-PTP α* cDNA (Elson and Leder, 1995a). The intron-exon structure of selected regions of these clones was determined and was found to be identical to that of *PTP α* exons 12–17 (Wong et al., 1993), with the PTPase signature motif spanning exons analogous to *PTP α* exons 13 and 14 (not shown). The targeting construct (Figure 1A) was based on the pPNT vector (Tybulewicz et al., 1991), with the Neo^r expression cassette of pPNT replacing a 2.9 kb *PTP α* genomic sequence containing exons 13–15. Following linearization and electroporation into TC1 ES cells (Deng et al., 1996), genomic DNA from ES cell clones that survived G418 and FIAU selection was analyzed by Southern blotting and PCR (not shown). Cells from two ES cell clones heterozygous for the targeted mutation were injected into C57BL/6 blastocysts, leading to chimeric mice and subsequently to germline transmission of the mutant allele. Mice were genotyped using DNA from tail biopsies by the Southern blot technique as shown in Figure 1B. All mice used in this report were of a mixed 129×C57BL/6 background.

Cell culture

Primary Schwann cells were prepared from 3- to 5-day-old WT (*Ptpre*^{+/+}) or *Ptpre*^{-/-} pups as described (Sobko et al., 1998a). The young age of the pups prevented their individual genotyping prior to cell preparation, hence age-matched WT and *Ptpre*^{-/-} pups were obtained from separate matings of WT or of *Ptpre*^{-/-} mice in which all pups were of the desired genotype. Sciatic nerves obtained from mice of a given litter were pooled prior to cell preparation. HEK 293 cells were grown and transfected by the calcium phosphate technique as described (Toledano-Katchalski et al., 1999). In some cases HEK 293 cells were transfected with vectors expressing rat Kv2.1 (gift of Drs J. Barhanin and M. Lazdunski), chicken Y527F Src and human Fyn Y531F (gifts of Dr S. Courtneidge), or mouse cyt-PTP α (Toledano-Katchalski et al., 1999).

Electrophysiology

Macroscopic whole-cell currents were recorded in Schwann cells or in transfected HEK 293 cells, as previously described (Sobko et al., 1998a). HEK 293 cells were co-transfected with pIRES-CD8 (kindly provided by Drs J. Barhanin and A. Patel, CNRS, Sophia Antipolis, France) as a marker for transfection. Transfected cells were visualized 40 h following transfection, using the anti-CD8 (Dyna) antibody-coated bead method (Jurman et al., 1994). Signals were amplified using an Axopatch 200B patch-clamp amplifier (Axon Instruments), sampled at 2 kHz and filtered below 0.8 kHz via a four-pole Bessel low pass filter. Data acquisition was carried out using pClamp 6.0.2 software (Axon Instruments). The patch pipettes with tip resistance of 4–8 M Ω were filled with (in mM): 164 KCl, 2 MgCl₂, 1 CaCl₂, 11 EGTA, 10 HEPES, 11 glucose at pH 7.4. The external solution contained (in mM): 140 NaCl, 5 KCl, 5 CaCl₂, 2 MgCl₂, 11 glucose and 10 HEPES at pH 7.4. Series resistances were within 8–16 M Ω and were compensated by 85–90%. The sustained component of the K⁺ currents was measured at the end of the depolarizing traces. All data were expressed as mean \pm SEM. Statistically significant differences were assessed by Student's *t*-test.

For heterologous expression in *Xenopus laevis*, stage V and VI oocytes were used to inject cRNA, as described previously (Abitbol et al., 1999). Capped complementary RNAs (cRNAs) were injected at 2.5 ng cRNA/oocyte. Standard two-electrode voltage-clamp measurements were performed 3–5 days following cRNA microinjection into oocytes as described (Abitbol et al., 1999). Current signals were filtered at 0.5 kHz and digitized at 2 kHz. Chord conductance (*G*) was calculated using the following equation: $G = I/(V - V_{rev})$ where *I* corresponds to the current amplitude and *V*_{rev} is the measured reversal potential, assumed to be -90 mV (-90 \pm 2 mV; *n* = 7). *G* was estimated at various test voltages *V* and then, normalized to a maximal conductance value *G*_{max}, calculated at +30 mV. Activation curves were fitted by a Boltzmann distribution: $G/G_{max} = 1/[1 + \exp[(V_{50} - V)/s]]$, where *V*₅₀ is the voltage at which the current is half-activated and *s* is the slope factor.

Protein blot analysis

Selected organs and primary Schwann cells were homogenized in buffer A (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1% NP-40), supplemented with 0.5 mM sodium pervanadate and protease inhibitors (Sigma), and analyzed as described (Elson and Leder, 1995a). Antibodies used in this study included polyclonal anti-PTP α (Elson and Leder, 1995a), monoclonal (clone D4/11) or polyclonal anti-Kv2.1 (Upstate Biotechnology), polyclonal anti-Kv1.5 (Upstate Biotechnology and Alomone Labs), monoclonal anti-Fyn (Santa Cruz Biotechnology), monoclonal anti-v-Src (Calbiochem), anti-FLAG M2 affinity beads

(Sigma) or anti-phosphotyrosine (clone PY20; Transduction Laboratories).

Immunoprecipitation and substrate-trapping experiments

The D245A mutation was introduced into the mouse cyt-PTP α cDNA (Elson and Leder, 1995b) by site-directed mutagenesis; the presence of the desired mutation and absence of other mutations were verified by DNA sequencing. PTP α cDNAs all contained a FLAG tag at their C-termini and were expressed from the pCDNA3 vector (Invitrogen). Wild-type cyt-PTP α , but not D245A cyt-PTP α , was catalytically active towards paranitrophenyl phosphate (not shown). For immunoprecipitations, cells were lysed in buffer A supplemented with 5 mM iodoacetic acid and protease inhibitors. Cellular proteins (0.5–1 mg) were reacted with the relevant antibodies for 6–8 h, followed by four extensive washes with RIPA buffer. When used, pervanadate (0.5 mM) replaced iodoacetate in the lysis buffer.

Electron microscopy

Three 5-day-old mice of either genotype were studied. Nerve fibers were fixed in 3% paraformaldehyde/2% glutaraldehyde and processed for electron microscope analysis as described (Shinder and Devor, 1994). Samples from different mice were always taken from the same location, 5 mm from the proximal end of the nerve. Vertical cross-sections 70–90 nm thick were cut with a Leica Ultracut UCT ultra-microtome, stained with uranyl acetate and lead citrate, and examined in a Philips 410 transmission electron microscope at 100Kv. Five to seven non-overlapping cross-section fields were examined from each mouse at 2800 \times magnification. The thicknesses of myelin sheaths of all axon profiles in a given field were measured, and data for all mice of the same genotype were pooled. Similar experiments were conducted on sciatic nerve tissue obtained from 8-month-old male mice (three mice from either genotype).

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